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THE ACTIVATION OF INTRACELLULAR PROTEINASES

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Preparations of intracellular proteinases, as obtained from plant or animal material, usually contain natural activators. In the present communication it is shown that the response of intracellular proteinases to added activators may be different, depending upon the presence or absence of natural activators. Even minute quantities of natural activators may influence decisively the course of the activation. For example, it has been found that the most widely studied activation phenomenon, the activation of papain by HCN, is due to the presence of traces of natural activators and that the removal of these natural activators through careful dialysis renders the papain unactivatable by HCN.

Activation of Papain by HCN - A solution of papain was thoroughly dialyzed, as described in the experimental section. A solution of this dialyzed papain was found to be inactive toward benzoyl-L-arginineamide when no activator was added. After addition of HCN, no increase in enzymatic activity was observed. However, when a very small quantity of H₂S, cysteine, or glutathione was added to the dialyzed enzyme solution together with the HCN, a high activity toward benzoylarginineamide resulted. These small quantities of sulfhydryl compounds, when employed in the absence of HCN, were insufficient to cause any activation of the papain (Table I).

Furthermore, the activation of the dialyzed papain by HCN in the presence of traces of cysteine was found to be reversed when the HCN was removed *in vacuo*. After evacuation, the enzyme solution was inactive toward benzoylarginineamide. Addition of fresh HCN restored the activity (Table II).

The fact that dialysis removes from papain a substance essential for HCN activation of the papain is shown by the following experiment. The dialysate obtained in preparing the dialyzed papain was concentrated to a small volume. When this concentrated dialysate and the dialyzed papain were mixed in the original proportions and the mixture was treated with HCN, the

TABLE I

HCN-Activation of Papain. Effect of Traces of Sulfhydryl Compounds

Substrate, benzoyl-*l*-arginineamide, enzyme, dialyzed papain, 0.0166 mg. of protein N per cc. of test solution. Temperature, 40°; pH 5.1 to 5.3.

| Activator | Activator concentration in test solution | $K \times 10^4$ * |
|------------------|------------------------------------------|-------------------|
| | <i>mM per cc.</i> | |
| None | 0 | 0† |
| HCN | 0.020 | 0-5 |
| Cysteine | 0.00004 | 0† |
| Cysteine | 0.00004 | 20 |
| HCN | 0.020 | |
| H ₂ S | 0.00004 | |
| H ₂ S | 0.00004 | 19 |
| HCN | 0.020 | |
| Glutathione | 0.00004 | |
| Glutathione | 0.00004 | 31 |
| HCN | 0.020 | |
| Cysteine | 0.020 | |
| H ₂ S | 0.016 | 12 |
| Glutathione | 0.020 | 30 |

* $K = 1/t \log (a)/(a-x)$. Each value of K in this and in the following tables represents the average of several constants calculated, as previously described (1).

† These values were determined by carrying out all operations in the complete absence of oxygen to prevent oxidation of the trace of sulfhydryl compound added. In the remaining experiments, no precautions were taken to avoid oxidation. Consequently, the possibility exists that the effective concentration of the sulfhydryl compounds in the latter experiments might be even less than that indicated above.

resulting enzyme solution was highly active. The subsequent removal of HCN *in vacuo* resulted in an inactivation (Table II).

As was to be expected from the preceding experiment, a sample of the original undialyzed papain became highly active on addition of HCN and lost its activity on subsequent removal of the HCN *in vacuo* (Table II).

From these experiments it must be concluded that papain¹ exists in two forms: one of these (α -papain) is not activatable by HCN but may be transformed by SH compounds into another form (β -papain) that is activatable by HCN. The fact that this

TABLE II

HCN-Activation of Papain. Effect of Removal of HCN in Vacuo
Substrate, benzoyl-*L*-arginineamide. Temperature, 40°; pH 5.1 to 5.3.

| Enzyme preparation | Enzyme concentration in test solution | Treatment* | $K \times 10^4$ |
|------------------------------|---------------------------------------|--------------------------------------|-----------------|
| | <i>mg. protein N per cc.</i> | | |
| Dialyzed papain | 0.0166 | (a) None | 0 |
| | 0.0166 | (b) Cysteine (0.00004) + HCN (0.020) | 32 |
| | 0.0166 | (c) (b) after evacuation | 0 |
| | 0.0166 | (d) (c) + HCN (0.025) | 31 |
| | 0.0166 | (e) Cysteine (0.020) | 33 |
| Dialyzed papain + dialysate† | 0.0432 | (a) None | 0 |
| | 0.0155 | (b) HCN (0.020) | 17 |
| | 0.0155 | (c) (b) after evacuation | 3 |
| | 0.0155 | (d) (c) + HCN (0.025) | 16 |
| | 0.0155 | (e) Cysteine (0.020) | 35 |
| Undialyzed papain‡ | 0.0165 | (a) None | 0 |
| | 0.0165 | (b) HCN (0.020) | 18 |
| | 0.0165 | (c) (b) after evacuation | 3 |
| | 0.0165 | (d) (c) + HCN (0.025) | 17 |
| | 0.0165 | (e) Cysteine (0.020) | 36 |
| Dialysate from papain | 0 | (a) HCN (0.020) | 0 |
| | 0 | (b) (a) after evacuation | 0 |
| | 0.0171 | (c) (b) + dialyzed papain§ | 0 |

* The figures in parentheses denote activator concentration in mM per cc. of test solution.

† Dialyzed enzyme and dialysate were mixed in the proportions originally present in the undialyzed enzyme.

‡ The procedure employed in this experiment has been described in detail in the experimental section.

§ The concentration of the treated dialysate used was equivalent to 4 times that originally present in the undialyzed papain.

¹ Since papain probably represents a mixture of enzymes, the conclusions drawn in this paper are valid only for the component of HCN-papain that hydrolyzes benzoylarginineamide. It is probable that other components of papain are activated through the formation of similar compounds with HCN.

activation by HCN may be reversed under conditions that exclude oxidation by atmospheric oxygen indicates that the HCN activation consists in the formation of a dissociable HCN- β -papain compound.

The activation of undialyzed papain by HCN is due to the presence of natural activators; it must be assumed that a small quantity of these natural activators is present in the SH form and that this trace acts in the same manner as shown above for minute amounts of H_2S , cysteine, or glutathione

It is obvious from the foregoing that if, on addition of HCN, a papain preparation acquires the ability to hydrolyze benzoyl-arginineamide it may be concluded that the papain preparation must contain at least a small quantity of a natural activator.

In a previous communication (2) it has been reported that papain becomes inactive when precipitated by means of isopropyl alcohol from a solution of HCN-activated papain. In the opinion of Greenberg and Winnick (3), this result is due to mild oxidation of the active papain by oxygen and not to the dissociation of an enzyme-activator compound. It is clear from the above experiments on the inactivation of HCN-papain by removal of HCN *in vacuo* that this conclusion of Greenberg and Winnick must be regarded as erroneous. Furthermore, in repeating the above experiment, Greenberg and Winnick activated the papain before precipitation, not with HCN, but with cysteine. It might be expected that under these conditions a sufficient quantity of cysteine would be precipitated with the papain to activate the redissolved papain.

Activation of Papain by H_2S A solution of dialyzed papain was inactive toward benzoylarginineamide but became active toward this substrate after treatment with H_2S . When the enzyme solution was freed of H_2S by evacuation, the resulting solution became nearly inactive toward the substrate. Addition of fresh H_2S restored the activity completely (Table III).

However, when the undialyzed papain was activated by H_2S , the subsequent removal of H_2S *in vacuo* caused no loss in activity. Identical results were obtained when the same procedure was applied to a mixture of the dialyzed papain and its dialysate (Table III). The difference in the behavior of dialyzed and undialyzed papain toward H_2S is apparently due to the fact that

undialyzed papain contains substances that are transformed into activators by H_2S . These substances are removed on dialysis. This conclusion was verified as follows: The concentrated dialysate was treated with H_2S and the H_2S was then removed by evacuation. Following this procedure the treated dialysate was added

TABLE III

H₂S-Activation of Papain. Effect of Removal of H₂S in Vacuo

Substrate, benzoyl-L-arginineamide. Temperature, 40°; pH 5.1 to 5.3.

| Enzyme preparation | Enzyme concentration in test solution | Treatment* | $K \times 10^4$ |
|------------------------------|---------------------------------------|----------------------------|-----------------|
| | <i>mg. protein N per cc.</i> | | |
| Dialyzed papain | 0.0302 | (a) None | 1 |
| | 0.0169 | (b) H_2S (0.016) | 19 |
| | 0.0169 | (c) (b) after evacuation | 3 |
| | 0.0169 | (d) (c) + H_2S (0.016) | 18 |
| Undialyzed papain | 0.0165 | (a) None | 0 |
| | 0.0165 | (b) H_2S (0.016) | 17 |
| | 0.0165 | (c) (b) after evacuation | 16 |
| | 0.0165 | (d) (c) + H_2S (0.016) | 15 |
| Dialyzed papain + dialysate† | 0.0302 | (a) None | 0 |
| | 0.0169 | (b) H_2S (0.016) | 14 |
| | 0.0169 | (c) (b) after evacuation | 17 |
| Dialysate from papain | 0 | (a) H_2S (0.016) | 0 |
| | 0 | (b) (a) after evacuation | 0 |
| | 0.0169 | (c) (b) + dialyzed papain‡ | 8-10 |

* The figures in parentheses denote activator concentration in mm per cc. of test solution.

† Dialyzed enzyme and dialysate were mixed in the proportions originally present in the undialyzed enzyme.

‡ The concentration of the treated dialysate used was equivalent to 4 times that originally present in the undialyzed papain. The activator formed by H_2S treatment of the dialysate is very rapidly destroyed on exposure to air.

to dialyzed papain and the activity of the mixture was determined. The high activity observed indicates that the dialysate contained a substance (or substances) that was transformed into an activator by H_2S (Table III).

Several years ago, Hellerman and Perkins (4) reported an experiment in which the activity of an H_2S -activated papain prepa-

ration was not changed by the removal of the H_2S in a stream of nitrogen. In view of the above experiments, it would appear that the papain preparation employed by these workers contained a potential activator.

From the experiments described in this section, it is apparent that the action of H_2S on dialyzed papain consists of two distinct steps. The first involves the transformation of α -papain into β -papain; the second, the activation of β -papain through the formation of an H_2S - β -papain compound. As mentioned in a previous paragraph, the amount of H_2S required to perform the first step is relatively small. However, a much larger amount of H_2S is required to convert β -papain completely into an active enzyme. From the reversible nature of the second step it must be concluded that the active enzyme represents a dissociable H_2S - β -papain compound. It will be recalled that HCN is not able to transform α -papain into β -papain, but can combine with β -papain to give an active enzyme. It may be postulated that sulfhydryl compounds such as cysteine or glutathione are, like H_2S , capable of transforming α -papain into β -papain and also of combining with β -papain to give an active enzyme.

When, however, potential activators are present in a papain solution, H_2S may perform an additional function in the activation process. As was shown above, H_2S may transform the potential natural activator into a product that in turn activates papain. This newly formed activator is not removed on evacuation. Therefore, when the activation of an intracellular enzyme by H_2S is found to be irreversible under the experimental conditions described in this communication, it may be concluded that the enzyme preparation contains a potential activator that is transformed by H_2S into a true activator.

Nature of the Dialyzable Potential Activator in Papain—When the concentrated dialysate obtained from papain was treated with HCN and the HCN was removed *in vacuo*, the resulting solution failed to activate dialyzed papain (Table II). This indicates that the potential activator present in the dialysate is not transformed into a true activator by HCN . Apparently the chemical nature of the dialyzable potential activator present in crude papain preparations is such that it is transformed into a true activator by H_2S but not by HCN .

Numerous investigators, in particular Grassmann (5) and

Maschmann and Helmert (6), have discussed the presence of disulfide and sulfhydryl compounds in crude papain and their possible rôle as natural activators of the enzyme. Grassmann (7) assumed that HCN activates papain only indirectly. He suggested that there is present in papain a substance that is transformed by HCN into a sulfhydryl compound which in turn activates papain. As experimental support for his view, Grassmann mentioned his observation that the disulfide form of glutathione is reduced to the sulfhydryl form by means of KCN.

It seems necessary to emphasize, however, that the reduction of SS-glutathione by cyanide has been performed only at rather alkaline pH values, while the HCN activation of papain takes place at pH 5. When the disulfide form of glutathione is treated with HCN at pH 5 and the HCN is then removed *in vacuo* with exclusion of oxygen, the resulting solution does not activate dialyzed papain for the hydrolysis of benzoylarginineamide. In other words, HCN does not produce from SS-glutathione at pH 5 a quantity of SH-glutathione sufficient for the activation of papain (Table IV).

When SS-glutathione is treated with H_2S at pH 5, and the H_2S is then removed by evacuation, the resulting solution is capable of activating papain for the hydrolysis of benzoylarginineamide (Table IV). It may be concluded, therefore, that, in contrast to the action of HCN, H_2S forms sufficient SH-glutathione to activate papain. Thus SS-glutathione and the dialyzable potential activator from papain resemble one another in their behavior toward HCN and H_2S at pH 5. It appears possible that the potential activator removed on dialysis of papain is a disulfide.

Activation of Cathepsin by HCN—Beef spleen cathepsin contains an enzymatic component which, on activation by HCN, splits benzoylarginineamide. The following experiments show that the activation mechanism for this component is similar to that demonstrated above for the HCN activation of papain. The activated cathepsin component may be designated "HCN-cathepsin (benzoylarginineamide)." Although it is probable that this cathepsin component is identical with Cathepsin II,² the above

² Cathepsin II is the enzymatic component of beef spleen extracts that hydrolyzes benzoylarginineamide when cysteine has been added as activator (8).

designation should be employed until experimental proof for the identity has been secured.

A carefully dialyzed solution of beef spleen cathepsin was inactive toward benzoylarginineamide. Furthermore, on addition of HCN only a slight activity resulted. When a very small quantity of cysteine was added together with HCN, a rapid

TABLE IV

Action of HCN and of H₂S on SS-Glutathione at pH 5

Substrate, benzoyl-L-arginineamide; enzyme, dialyzed papain, 0.0171 mg. of protein N per cc. of test solution. Temperature, 40°; pH of test solutions 5.1 to 5.3.

The reagents were prepared in the absence of the enzyme and substrate; the enzyme and substrate were then added and the values of K_{BAA} were determined.

| Reagent* | pH of reagent solution | $K \times 10^4$ |
|--------------------------------------------------------|------------------------|-----------------|
| (a) SS-Glutathione (0.020) | | 0 |
| (b) " (0.005) + HCN (0.020)† | 5 13 | 14‡ |
| (c) (b) after evacuation | 5.27 | 0 |
| (d) (c) + HCN (0.025)† | 5 24 | 16‡ |
| (e) SS-Glutathione (0.005) + H ₂ S (0.020)† | 5 21 | 13 |
| (f) (e) after evacuation | 5 32 | 23 |
| (g) (f) + H ₂ S (0.020)† | 5 30 | 10 |
| (h) SH-Glutathione (0.010) | | 18 |
| (i) " (0.005) | | 14 |

* The figures in parentheses denote concentrations in mm per cc. of test solution.

† Incubated for 2 hours at 40° before addition of enzyme and substrate. In the case of (e), precipitated sulfur was removed from the incubated solution by centrifugation before tests were performed.

‡ The activation obtained in these cases is due to the presence of a trace of sulfhydryl in the oxidized glutathione. The amount of sulfhydryl present is less than 0.00004 mm per cc. of test solution, as is indicated by the results given in Table I.

hydrolysis of the substrate was observed. This small quantity of cysteine, when employed in the absence of HCN, was insufficient to cause activation of beef spleen cathepsin (Table V). These results indicate that a small amount of cysteine suffices to transform this cathepsin component from an α form (unactivatable by HCN) into a β form (activatable by HCN).

It was found that this β form is inactivated by removal of HCN *in vacuo*. Addition of fresh HCN to the inactive solution restores the enzymatic activity toward benzoylarginineamide (Table V).

Reduction Theory of Activation of Intracellular Proteinases—The activation of the intracellular proteinases has been regarded as a reduction process. The activatable enzymes were believed to be reversibly reducible and oxidizable and to be hydrolytically active only in the reduced state. The reduction and oxidation were supposed to represent the appearance and disappearance of a sulfhydryl group in the enzyme (9). This theory rests upon the experimental finding that active papain solutions may be in-

TABLE V
Activation of Beef Spleen Cathepsin

Substrate, benzoyl-*l*-arginineamide; enzyme, dialyzed cathepsin, 0.15 mg. of protein N per cc of test solution Temperature, 40°; pH 4.7.

| Treatment* | $K \times 10^4$ |
|--------------------------------------|-----------------|
| (a) None | 0 |
| (b) Cysteine (0.020) | 17 |
| (c) " (0.00004) | 0 |
| (d) HCN (0.020) | 2 |
| (e) Cysteine (0.00004) + HCN (0.020) | 14 |
| (f) (e) after evacuation | 0 |
| (g) (f) + HCN (0.025) | 14 |

* The figures in parentheses denote activator concentration in mm per cc. of test solution.

activated by oxidizing agents and may be reactivated by reducing agents (Bersin (10), Hellerman (11), and Purr (12)). However, in these experiments the enzyme preparations had not been completely freed of accompanying natural activators and it was not demonstrated whether the oxidation-reduction procedures employed had oxidized or reduced the enzyme itself or the accompanying natural activators. On the other hand, it has been shown above that the activation of papain and of cathepsin by HCN is reversible under conditions in which oxidation and reduction do not occur. Consequently, when an activatable enzyme of the papain-cathepsin group is inactivated by oxidation procedures and reactivated by reduction procedures, it may be concluded

that the observed effects are due to the oxidation or reduction of accompanying activators.

It was previously shown that the transformation of papain into an active enzyme involves two distinct steps: (1) the conversion of inactive α -papain into inactive β -papain, and (2) the activation process itself, consisting of the formation of an activator- β -papain compound. It should be emphasized that the preceding discussion of the activation process applies only to the second step. It has not been determined whether or not oxidation-reduction processes are involved in the first step.

EXPERIMENTAL

Preparation of Enzymes

Preparation of "Undialyzed" Papain—A good grade of vacuum-dried papaya latex was purified by a method similar to that described by Grassmann (5). 250 gm. of finely ground latex were shaken for 1 hour with 2.5 liters of distilled water. The suspension was cooled to 4° and was filtered through a layer of Filter Cel. The filtrate was immersed in an ice bath and H_2S was passed rapidly through the solution for 6 hours. The solution was filtered rapidly with the aid of Filter Cel and to the clear filtrate (2620 cc.) was added sufficient 95 per cent methanol (7340 cc.) to make the alcohol concentration 70 per cent. The container was tightly stoppered to prevent loss of H_2S and the precipitated material was allowed to settle overnight in the cold. The precipitate was collected by filtration on a layer of Filter Cel and was washed three times with 70 per cent methanol by suspension and filtration. The washed precipitate was dissolved in water, filtered to remove the Filter Cel, and the filtrate (1160 cc.) was again treated with H_2S by the above procedure for 4 hours. Methanol (3080 cc.) was added to the clear filtrate (1100 cc.) to make the alcohol concentration 70 per cent, and the precipitate was collected by centrifugation after settling overnight in the cold. The precipitate was washed in closed centrifuge bottles twice with 70 per cent methanol, twice with absolute methanol, and once with ether. The ether was removed by repeated evacuation in the cold and the material was finally dried over P_2O_5 in a vacuum desiccator at room temperature. Yield, 83 gm. Nitrogen, 12.7 per cent; protein nitrogen, 8.3 per cent.

Preparation of "Dialyzed" Papain—200 mg. of the above papain preparation were dialyzed against distilled water at 4° for 7 days in a Kunitz dialyzer. The dialyzed enzyme was finally diluted with 12.5 cc. of citrate buffer (0.2 M, pH 5) and distilled water to 50 cc. (0.336 mg. of protein N per cc., 0.336 mg. of total N per cc.). The dialyzed enzyme solution was carefully sealed and stored in a desiccator containing lead acetate solution to prevent contamination by traces of hydrogen sulfide. The dialysate obtained in the above procedure was concentrated *in vacuo* (0.32 mg. of N per cc.; pH of solution, 6.8).

Preparation of Dialyzed Cathepsin—Beef spleen cathepsin was prepared as described in a previous communication (13) and was dialyzed at 4° against distilled water for 7 days. The resulting solution contained 1.08 mg. of protein N per cc.

Methods

The substrate, benzoyl-L-arginineamide hydrochloride, was prepared as previously described (14). The rate of hydrolysis was determined by the method of Grassmann and Heyde (15). Each test was performed as follows: 1.0 cc. of an aqueous solution of benzoylarginineamide hydrochloride (41.5 mg. per cc.) and 0.25 cc. of 0.2 M citrate buffer (pH 5.0) were warmed to 40° in a 2.5 cc. volumetric flask. The desired volumes of enzyme and activator solutions were then added, the mixture was diluted to 2.5 cc. with water at 40°, and the incubation was begun. In experiments in which H₂S or HCN was used, the enzyme, activator, and buffer were first incubated for 2 hours at 40° before addition of the substrate. However, in experiments in which cysteine or glutathione alone was used as the activator, the enzyme, activator, buffer, and substrate were mixed as described above, and measurement of the rate of hydrolysis was begun immediately.

Activator solutions were freshly prepared immediately before use. Cysteine hydrochloride and glutathione solutions were prepared by dissolving the analytically pure compounds in distilled water with sufficient N NaOH to give a pH of 5.0. A solution of HCN at pH 5.0 was prepared by adding the proper amount of N HCl to an ice-cold solution of KCN. A saturated solution of H₂S (approximately 0.2 M) was prepared by passing the gas into ice-cold, oxygen-free, distilled water. The disulfide form of

glutathione was prepared by treating an ice-cold, neutral solution of pure glutathione with 1 equivalent of hydrogen peroxide. The reaction mixture was shaken for a few seconds and was then evaporated to dryness. The residue was dissolved in water and the resulting solution was adjusted to pH 5.0. Tests for sulfhydryl and peroxide in this solution were negative.

The apparatus used for the removal *in vacuo* of volatile activators from enzyme solutions is shown in Fig. 1.

Nitrogen was purified by passing the gas over hot, reduced copper gauze. The apparatus from *A* to *D* was freed of oxygen by repeatedly evacuating the system and refilling it with nitrogen. During evacuation the opening at *B* was closed and the 2-way stop-cock at *A* was turned to permit the escape of nitrogen through the

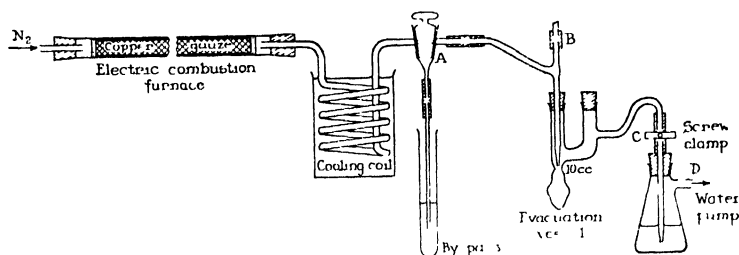


FIG. 1. Apparatus for evacuation of enzyme solutions in an atmosphere of nitrogen. *A*, 2-way stop-cock, *B*, inlet tube; *C*, screw-clamp

by-pass. After evacuation the clamp at *C* was closed and nitrogen was admitted to the system by turning the stop-cock *A*. Nitrogen was then allowed to escape through the opening at *B* for a few minutes, *C* was opened, and the test solution (usually 5.0 cc.) was introduced through *B* by means of a pipette. The solution was washed into the vessel by means of oxygen-free distilled water (2.0 cc.), a drop of octyl alcohol was added to reduce foaming, and the opening at *B* was closed. The stop-cock at *A* was turned to the by-pass and the system was carefully evacuated to remove the volatile activator. The trap flask contained 20 per cent lead acetate solution in the H_2S experiments and 0.5 *N* silver nitrate solution in the HCN experiments. After most of the dissolved gas had been removed, the evacuation vessel was immersed in a water bath and the temperature was raised to 35–40°.

Evacuation at approximately 15 mm. was continued for $1\frac{1}{2}$ hours at this temperature. At the end of this period the system was filled with nitrogen, as described above, and oxygen-free water was added through *B* to dilute the small residue (approximately 0.5 cc.) to the 10.0 cc. mark. The vessel was removed from the apparatus, stoppered tightly, and the contents were mixed. Portions of this solution were then tested for enzymatic activity and treated as indicated in Tables II to V. Test experiments were carried out with papain, activated by such amounts of cysteine that oxidation would have resulted in a decrease of the velocity constant. Under the conditions described above, no decrease in the velocity constant was observed.

A detailed description of the procedure followed in carrying out a typical evacuation experiment (undialyzed papain, Table II) is given below. The numbers used to designate the steps in the procedure correspond to those given in Table II.

50 mg. of undialyzed papain (4.126 mg. of protein nitrogen) were dissolved in 6.25 cc. of 0.2 M citrate buffer, pH 5.0, and enough distilled water to make the volume 25.0 cc. The activity of this solution (*a*) toward benzoyl-*L*-arginineamide was determined as previously described, 0.25 cc. of the enzyme being used in the 2.5 cc. test flask. The activity of the enzyme in the presence of an excess of cysteine (*e*) was determined in the same manner on another 0.25 cc. portion of the enzyme, 0.5 cc. of 0.1 M cysteine solution (78.8 mg. of cysteine hydrochloride + 0.4 cc. of N NaOH in 5.0 cc.) being used as the activator.

12.5 cc. of the untreated papain (Solution *a*) were treated with 10 cc. of 0.25 M HCN solution (407 mg. of KCN dissolved in 15 cc. of distilled water in an ice bath, plus 6.0 cc. of N HCl, diluted to 25.0 cc.). The mixture was diluted to 25.0 cc. with citrate buffer and was incubated for 2 hours at 40°. The velocity constant (K_{BAA}) was determined as before, with 0.5 cc. of this enzyme solution (*b*). 5.0 cc. of the HCN-activated papain (*b*) were evacuated and diluted to 10.0 cc. as described above. The K_{BAA} was determined with 1.0 cc. of this enzyme solution (*c*). 4.0 cc. of solution (*c*) were then treated with 1.0 cc. of 0.25 M HCN and the mixture was incubated for 2 hours at 40°. The reactivated enzyme was again tested on 1.25 cc. of this enzyme solution (*d*).

In all evacuation experiments controls were run which showed

that the change in activity was due to the evacuation and not to other experimental conditions.

SUMMARY

The activation of the components of papain and of beef spleen cathepsin that hydrolyze benzoyl-*L*-arginineamide has been studied by means of reaction kinetics. Each of these components has been found to exist in two inactive forms (α and β form). The α form is not activated by HCN, but may be converted into the β form which is then activated by HCN. The activation of the β form by HCN or H₂S consists in the formation of dissociable compounds of the β form with the activator. Furthermore, the activation and inactivation of the β form can be accomplished without the mutual transformation of SS and SH groups and without the occurrence of reduction and oxidation processes.

The natural activators usually present in papain and cathepsin preparations have been found to influence the effect of added activators in several ways. In order to obtain unambiguous information about the effect of added activators, the natural activators must be removed.

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CONFIGURATIONAL RELATIONSHIP OF 2-METHYL- HEPTANOIC AND 4-METHYLNONANOIC ACIDS

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The configuration of 2-methylheptanoic acid has not previously been correlated to that of 3-methyloctanoic and 4-methylnonanoic acids.

This has now been accomplished by a series of reactions shown in Formulas I to V. A malonic ester synthesis from levo-1-iodo-2-methylheptane (IV) yielded levo-4-methylnonanoic acid (V). Previously, Levene and Marker¹ made the same acid from dextro-3-methyloctanoic acid. Thus levo-2-methylheptanoic, dextro-3-methyloctanoic, and levo-4-methylnonanoic acids are configurationally related. The relationship is the same as that found in the methyl-*n*-propyl and methyl-*n*-butyl series by Levene and Marker.²

EXPERIMENTAL

Resolution of 2-Methylheptanoic Acid (Methyl Amyl Acetic Acid)
—900 gm. of *dl*-2-methylheptanoic acid were neutralized with 1825 gm. of cinchonidine in acetone. The mixture was filtered, and the mother liquor was evaporated under reduced pressure and the acid recovered. B.p. 94–96°, 1 mm.; yield, 150 gm.; $n_D^{25} = 1.4235$.

$$[\alpha]_D^{25} = +8.9^\circ \text{ (homogeneous)}$$

The crystals were recrystallized from acetone six times, and from methyl ethyl ketone three times more. The acid was extracted and distilled. Yield, 50 gm.; $d_4^{25} = 0.902$ (*in vacuo*); $n_D^{25} = 1.4233$.

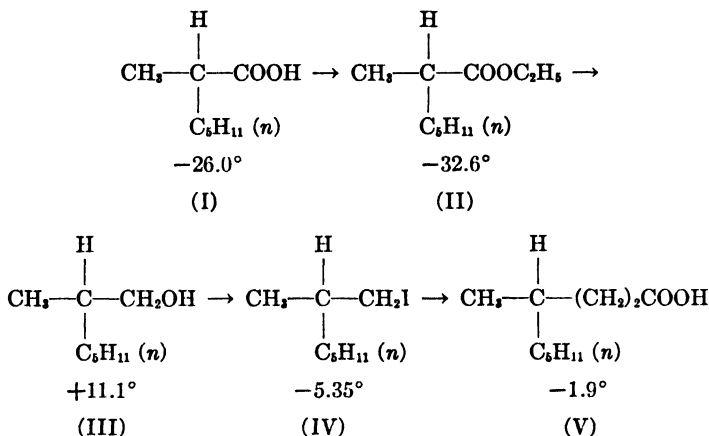
$C_9H_{18}O_2$. Calculated, C 66.63, H 11.19; found, C 66.56, H 11.35

* Died September 6, 1940.

¹ Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **95**, 1 (1932).

² Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **103**, 299 (1933).

$[\alpha]_D^{25} = -15.6^\circ$; $[M]_D^{25} = -22.5^\circ$, *i.e.* 86.5 per cent of the maximum $[M]_D^{25} = -26.0^\circ$ (homogeneous), calculated from the maximum rotation of 4-methylnonanoic acid given by Levene and Marker,³ assuming no racemization in reactions (I) to (V).



Dextro-2-Methylheptanoic Ethyl Ester—236 gm. of 2-methylheptanoic acid, $[\alpha]_D^{25} = +8.48^\circ$ (homogeneous), *i.e.* 47 per cent of the maximal value, were dissolved in 300 gm. of dry ethanol containing 15 gm. of concentrated sulfuric acid. The solution was refluxed for 2 hours. The ester was isolated as usual. B.p. 80° , 15 mm.; yield, 246 gm.; $d_4^{25} = 0.860$ (*in vacuo*); $n_D^{25} = 1.4119$

$\text{C}_{10}\text{H}_{20}\text{O}_2$. Calculated, C 69.72, H 11.70; found, C 69.77, H 11.87
 $[\alpha]_D^{25} = +8.91^\circ$; $[M]_D^{25} = +15.3^\circ$, *i.e.* 47% of calculated maximum $[M]_D^{25} = +32.6^\circ$ (homogeneous)

Levo-2-Methyl-1-Heptanol—246 gm. of 2-methylheptanoic ethyl ester, $[\alpha]_D^{25} = +8.91^\circ$ (homogeneous), were reduced in the following manner. 50 gm. of sodium were melted in toluene, broken into fine pellets with a stirrer, and let cool. The toluene was poured off and the sodium particles were washed several times with dry hexane. Then 250 cc. of dry hexane were added and 30 gm. of the ester dissolved in 30 cc. of dry methanol were slowly added with rapid stirring. 400 cc. of dry methanol were then added in such

³ Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **95**, 162 (1932).

a manner as to continue refluxing until all of the sodium was dissolved. 500 cc. of water were then added, and the carbinol was extracted with ether. The extract was washed with water and dried with anhydrous potassium carbonate. The carbinol distilled at 85–89°, 18 mm.; yield, 106 gm.; $d_4^{25} = 0.823$ (*in vacuo*), $n_D^{25} = 1.4256$.

$C_8H_{18}O$. Calculated, C 73.78, H 13.93, found, C 73.73, H 14.00
 $[\alpha]_D^{25} = -4.01^\circ$; $[M]_D^{25} = -5.22^\circ$, i.e. 47% of calculated maximum $[M]_D^{25} = -11.1^\circ$ (homogeneous)

Dextro-1-Iodo-2-Methylheptane—106 gm. of 2-methyl-1-heptanol, $[\alpha]_D^{25} = -4.01^\circ$ (homogeneous), were transferred into five bomb tubes and 1.5 volumes of anhydrous hydrogen iodide were distilled into each. The sealed tubes were allowed to stand at 25° for 1 week, and were then heated for 1 hour at 60°. The iodide was isolated as usual.⁴ B.p. 78–80°, 8 mm.; yield, 167 gm.; $n_D^{25} = 1.4875$; $d_4^{25} = 1.333$ (*in vacuo*).

$C_8H_{17}I$. Calculated, C 39.98, H 7.13, found, C 40.00, H 7.07

$[\alpha]_D^{25} = +1.05^\circ$; $[M]_D^{25} = +2.52^\circ$, i.e. 47 per cent of the calculated maximum $[M]_D^{25} = +5.37^\circ$ (homogeneous), assuming no racemization.

*Dextro-4-Methylnonanoic Acid*⁵—160 gm. of 1-iodo-2-methylheptane, $[\alpha]_D^{25} = +1.05^\circ$ (homogeneous), were added to a solution which contained 14.7 gm. of sodium dissolved in 150 cc. of dry ethanol, and 112 gm. of ethyl malonate. After the mixture had been refluxed for 1.5 hours, the reaction was neutral. The ester was isolated as usual. After a forerun up to 210°, 760 mm., it distilled at 88–98°, 1 mm. Yield, 150 gm. The ester was hydrolyzed and the acid isolated as usual. B.p. 92°, 0.1 mm.; yield, 39 gm.

$C_{10}H_{20}O_2$. Calculated. C 69.72, H 11.70, mol. wt. 172.3

Found. " 69.81, " 11.84, " " 172.8

$[\alpha]_D^{25} = +0.46^\circ$; $[M]_D^{25} = +0.89^\circ$ (homogeneous)

Levene and Marker³ report a maximum $[M]_D^{25} = +1.9^\circ$ for this acid. The acid therefore contains 47 per cent of the dextro form.

⁴ Levene, P. A., and Rothen, A., *J. Biol. Chem.*, **115**, 415 (1936).

⁵ Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **95**, 17 (1932).

CONFIGURATIONAL RELATIONSHIPS OF ALIPHATIC AMINES

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In a previous publication¹ it was shown that members of a homologous series of normal aliphatic disubstituted acetic acids containing a methyl group have the same sign of rotation. The corresponding free amines when configurationally related have the opposite sign of rotation. From Table I, levo-2-methylheptanoic acid is correlated to dextro-1-amino-2-methylheptane and agrees with the other members of its series.

When the disubstituted acid contains an ethyl group, however, the acid and the free amine have the same sign of rotation. Thus, from Table I, levo-2-ethylcaproic acid leads to levo-1-amino-2-ethylhexane. The rotatory dispersion of the latter has been previously determined.²

In Table II the derivatives of 4-nonanol are given. These have been discussed in a previous publication² and the rotatory dispersion of the dextro-4-aminononane and its salt has been determined. The details, however, were omitted.

It is interesting to note that the reduction of dextro-5-methyldecanonitrile led to an inactive 1-amino-5-methyldecane. This is analogous to the case of an inactive 5-methyl-1-nonanol leading to an active bromide.³

The rotations of the configurationally related aliphatic amines are given in Table III.

* Died September 6, 1940.

¹ Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **95**, 153 (1932).

² Levene, P. A., Rothen, A., and Kuna, M., *J. Biol. Chem.*, **120**, 759 (1937).

³ Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **103**, 299 (1933).

EXPERIMENTAL

Levo-2-Methylheptanoyl Chloride—15 gm. of 2-methylheptanoic acid,⁴ $[\alpha]_D^{25} = -7.8^\circ$ (homogeneous), i.e. 43 per cent of the maximal value,⁵ were dissolved in 50 gm. of thionyl chloride. The mixture

TABLE I
Derivatives of Substituted Acetic Acids

$[M]_D^{25}$ homogeneous unless otherwise specified.

| Series | —COOH | —COCl | —CN | —CH ₂ NH ₂ | —CH ₂ NH ₂ · HCl (in H ₂ O) |
|-------------------------------------------------------------------------------------------------------------------------|---------|-------|--------|----------------------------------|--------------------------------------------------------------------|
| $\begin{array}{c} \text{H} \\ \\ \text{CH}_3-\text{C}- \\ \\ \text{C}_6\text{H}_{11} \text{ (n)} \end{array}$ | -26.0° | -21° | -43.3° | +14.5° | +12.2° |
| $\begin{array}{c} \text{H} \\ \\ \text{C}_2\text{H}_5-\text{C}- \\ \\ \text{C}_4\text{H}_9 \text{ (n)} \end{array}$ | -23.5°* | -12° | -27° | -3.1° | -8.1° |

* This value was calculated from the maximum rotation of the hydrocarbon methyl ethyl *n*-butylmethane to which the acid was converted, assuming no racemization (see foot-note 8).

TABLE II
Derivatives of 4-Nonanol

$[M]_D^{25}$ homogeneous unless otherwise specified.

| Carbinol | Iodide | Azide | Free amine* | Hydrochloride* (in H ₂ O) |
|----------|--------|-------|-------------|-----------------------------------------|
| +0.82° | -4 37° | -0.2° | +0.75° | -1.69° |

* See foot-note 2.

was allowed to stand at 25° for 1 hour, refluxed for 15 minutes, and then distilled. B.p. 67–70°, 12 mm.; yield, 15.5 gm.; $d_4^{25} = 0.94$.

$\text{C}_8\text{H}_{15}\text{OCl}$. Calculated, C 59.07, H 9.30; found, C 58.90, H 9.17
 $[\alpha]_D^{25} = -5.1^\circ$; $[M]_D^{25} = -9.1^\circ$; least maximum $[M]_D^{25} = -21^\circ$ (homogeneous)
 (The least maximum rotation is defined as the minimum possible value (see foot-note 8).)

⁴ Levene, P. A., and Kuna, M., *J. Biol. Chem.*, **140**, 255 (1941).

⁵ Calculated from the value of $[M]_D^{25} = -26.0^\circ$ as maximum (see foot-note 4).

Considerable racemization takes place during the above reaction. A sample of the same acid, when allowed to stand with thionyl chloride at room temperature overnight, and then refluxed for 15 minutes, gave an acid chloride of only $[\alpha]_D^{25} = -1.3^\circ$ (homogeneous). Because of this racemization, the calculated maximum rotations are given as the least maximum values.

Levo-2-Methylheptonitrile—15 gm. of 2-methylheptanoyl chloride, $[\alpha]_D^{25} = -5.1^\circ$ (homogeneous), were slowly dropped into

TABLE III
Configurationally Related Amines

$[M]_D^{25}$ (approximate maximum values).

| | R | $-\text{NH}_2$ | $-\text{CH}_2\text{NH}_2$ | $-(\text{CH}_2)_2\text{NH}_2$ | $-(\text{CH}_2)_3\text{NH}_2$ | $-(\text{CH}_2)_4\text{NH}_2$ | $-(\text{CH}_2)_5\text{NH}_2$ |
|-----------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------|----------------------------------------------|----------------------------------------------------|-------------------------------------------------------------|---------------------------------------------|------------------------------------------|-------------------------------|
| $\begin{array}{c} \text{CH}_3 \\ \\ \text{H}-\text{C}- \\ \\ \text{R} \end{array}$ | Ethyl <i>n</i> -Propyl <i>n</i> -Butyl <i>n</i> -Amyl <i>n</i> -Hexyl | $+0.7^\circ$ $+7.8^\circ$ $+6.8^\circ$ | -5.1° -14° -16° Levo | $+11^\circ$ -0.4° -1.7° -3.6° | $+12^\circ$ -0.7° -0.8° | $+16^\circ$ $+0.5^\circ$ 0° | $+2.4^\circ$ |
| $\begin{array}{c} \text{C}_2\text{H}_5 \\ \\ \text{H}-\text{C}- \\ \\ \text{R} \end{array}$ | <i>n</i> -Butyl <i>n</i> -Hexyl | $+4.2^\circ$ $+7.2^\circ$ | $+3.1^\circ$ | | | | |
| $\begin{array}{c} \text{C}_3\text{H}_7 (n) \\ \\ \text{H}-\text{C}- \\ \\ \text{R} \end{array}$ | <i>n</i> -Butyl <i>n</i> -Amyl <i>n</i> -Hexyl | $+0.6^\circ$ $+0.7^\circ$ Dextro | | | | | |

50 cc. of a 40 per cent aqueous solution of ammonia which was cooled to -10° . The amide was filtered and dissolved in benzene. The solution was evaporated to dryness under reduced pressure and the process repeated until the amide was dry. Then 40 gm. of thionyl chloride were added and the solution was refluxed for 45 minutes. The excess thionyl chloride was distilled off at atmospheric pressure. The nitrile distilled at $71-73^\circ$, 14 mm.; yield, 9 gm.; $d_4^{25} = 0.811$ (*in vacuo*); $n_D^{25} = 1.4131$.

$C_8H_{18}N$. Calculated, C 76.74, H 12.08; found, C 76.69, H 12.13
 $[\alpha]_D^{25} = -14.9^\circ$; $[M]_D^{25} = -18.6^\circ$; least maximum $[M]_D^{25} = -43.3^\circ$
 (homogeneous)

Dextro-1-Amino-2-Methylheptane—12 gm. of 2-methylheptano-nitrile, $[\alpha]_D^{25} = -9.37^\circ$ (homogeneous), were dissolved in 50 cc. of methanol, and 0.3 gm. of Adams catalyst was added. This was shaken in hydrogen at a pressure of 45 pounds (3 atmospheres) at room temperature for 2 days. The catalyst was filtered, and hydrogen chloride in methanol was added to the filtrate. This was evaporated to dryness under reduced pressure, and taken up in 50 per cent sodium hydroxide. The amine was extracted with ether. The extract was dried with sodium hydroxide pellets. On distillation, the residue gave two fractions, the expected amine which was a liquid in a dry ice-acetone bath and a higher fraction which proved to be the secondary amine, which crystallized in the cooling bath.

Fraction I—B.p. 105–106°, 113 mm.; weight, 5 gm.; $n_D^{25} = 1.4258$; $d_4^{25} = 0.777$ (*in vacuo*)

$C_8H_{18}N$. Calculated, C 74.34, H 14.82; found, C 74.17, H 15.03
 $[\alpha]_D^{25} = +3.04^\circ$; $[M]_D^{25} = +3.93^\circ$; least maximum $[M]_D^{25} = +14.5^\circ$
 (homogeneous)

$[\alpha]_D^{25} = +2.0^\circ$; $[M]_D^{25} = +3.3^\circ$; least maximum $[M]_D^{25} = +12.2^\circ$
 (hydrochloride 2.9% in H_2O)

Fraction II—B.p. 90–100°, 1 mm.; weight, 3 gm.; $n_D^{25} = 1.4380$; $d_4^{25} = 0.795$ (*in vacuo*)

$C_{10}H_{22}N$. Calculated, C 79.67, H 14.52; found, C 79.03, H 14.55
 $[\alpha]_D^{25} = +0.56^\circ$; $[M]_D^{25} = +1.35^\circ$; least maximum $[M]_D^{25} = +5.0^\circ$
 (homogeneous)

5-Methyldecanonitrile—7 gm. of 1-bromo-4-methylnonane,⁶ $[\alpha]_D^{27.5} = +2.5^\circ$ (homogeneous), *i.e.* 90 per cent of the maximal value,⁷ were added to a solution of 3 gm. of KCN in 7 cc. of water and 30 cc. of absolute ethanol. The solution was refluxed for 48 hours. The nitrile was isolated as usual. B.p. 106–110°, 11 mm.; yield, 4 gm.; $d_4^{25} = 0.8192$ (*in vacuo*); $n_D^{25} = 1.4317$.

$C_{11}H_{21}N$. Calculated, C 78.97, H 12.65; found, C 79.15, H 12.72
 $[\alpha]_D^{25} = +1.46^\circ$; $[M]_D^{25} = +2.44^\circ$; maximum $[M]_D^{25} = +2.71^\circ$ (homogeneous)

⁶ Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **91**, 77 (1931).

⁷ Calculated from the value of $[M]_D^{25} = +6.2^\circ$ as maximum (see footnote 3).

1-Amino-5-Methyldecane—4 gm. of 5-methyldecanonitrile, $[\alpha]_D^{25} = +1.46^\circ$ (homogeneous), were reduced with hydrogen at atmospheric pressure with Raney catalyst in methanol. The amine was isolated as usual. It was found to be optically inactive, and its hydrochloride was also inactive.

Levo-2-Ethylcaproyl Chloride—100 gm. of 2-ethylcaproic acid,⁸ $[\alpha]_D^{25} = -3.54^\circ$ (homogeneous), i.e. 22 per cent of the maximal value, were added to 200 gm. of thionyl chloride. The solution was allowed to stand overnight at room temperature, and was then heated for 1 hour on a steam bath. The excess thionyl chloride was distilled at reduced pressure. The residue was then distilled. B.p. $62-64^\circ$, 10 mm.; yield, 108 gm.; $n_D^{25} = 1.4294$; $d_4^{25} = 0.9414$ (*in vacuo*).

$C_8H_{16}OCl$. Calculated, C 59.07, H 9.30; found, C 58.85, H 9.10
 $[\alpha]_D^{25} = -1.63^\circ$, $[M]_D^{25} = -2.65^\circ$; least maximum $[M]_D^{25} = -12^\circ$
 (homogeneous)

Levo-2-Ethylhexanonitrile (Ethylbutylacetonitrile)—100 gm. of 2-ethylcaproyl chloride, $[\alpha]_D^{25} = -1.63^\circ$ (homogeneous), were added dropwise to 500 cc. of saturated alcoholic ammonia cooled in an ice-alcohol bath. The precipitate was filtered and washed with benzene. The filtrate was concentrated to dryness, and then dissolved in benzene, and again evaporated to dryness under reduced pressure. The residue was dissolved in 250 gm. of thionyl chloride and heated for 1 hour on a steam bath. The insoluble part was filtered, and the filtrate was distilled at reduced (10 mm.) pressure to remove the excess thionyl chloride (bath, 50°). The residue was then dissolved in ether and washed with water. The ether solution was dried with sodium sulfate. The nitrile was then distilled. B.p. $98-100^\circ$, 50 mm.; yield, 55 gm.; $n_D^{25} = 1.4148$; $d_4^{25} = 0.8057$ (*in vacuo*).

$C_8H_{16}N$. Calculated, C 76.74, H 12.08; found, C 76.92, H 12.05
 $[\alpha]_D^{25} = -4.80^\circ$, $[M]_D^{25} = -6.00^\circ$; least maximum $[M]_D^{25} = -27^\circ$
 (homogeneous)

*Levo-1-Amino-2-Ethylhexane (Ethylbutylethylamine)*²—23 gm. of 2-ethylhexanonitrile, $[\alpha]_D^{25} = -4.80^\circ$ (homogeneous), were dis-

⁸ Levene, P. A., Rothen, A., and Meyer, G. M., *J. Biol. Chem.*, **115**, 401 (1936).

solved in methanol, Raney catalyst added, and the mixture shaken in an atmosphere of hydrogen for 16 hours. The catalyst was filtered off, and methyl alcoholic hydrogen chloride was added to the filtrate, which was then concentrated to dryness under reduced pressure. The crystals were taken up in 50 per cent sodium hydroxide and the free amine was extracted with ether. The extract was dried with metallic sodium.

The amine distilled at 98–99°, 90 mm.; $n_D^{25} = 1.4286$; $d_4^{25} = 0.7844$ (*in vacuo*).

$C_8H_{19}N$. Calculated, C 74.34, H 14.82; found, C 74.20, H 14.71
 $[\alpha]_D^{25} = -0.52^\circ$; $[M]_{587.6}^{25} = -0.67^\circ$; least maximum $[M]_{587.6}^{25} = -3.1^\circ$
 (homogeneous)
 $[\alpha]_{587.6}^{25} = -1.07^\circ$; $[M]_{587.6}^{25} = -1.77^\circ$; least maximum $[M]_{587.6}^{25} = -8.1^\circ$
 (hydrochloride 33% in H_2O)

*Dextro-4-Nonanol*⁹—The inactive carbinol was prepared from *n*-amylmagnesium bromide and *n*-butyraldehyde in the usual manner. It was converted into the acid phthalic ester and this was neutralized with brucine in methyl isobutyl ketone. The salt was recrystallized nineteen times from methyl isobutyl ketone. The crystals yielded a carbinol; b.p. 94–95°, 18 mm.; $d_4^{25} = 0.8187$ (*in vacuo*); $n_D^{25} = 1.4275$.

$C_8H_{20}O$. Calculated, C 74.93, H 13.98; found, C 74.85, H 14.08
 $[\alpha]_D^{25} = +0.57^\circ$; $[M]_D^{25} = +0.82^\circ$ (homogeneous)
 $[\alpha]_D^{25} = +0.34^\circ$; $[M]_D^{25} = +0.49^\circ$ (9% in ether)

Levo-4-Iodononane—30 gm. of 4-nonanol, $[\alpha]_D^{25} = +0.57^\circ$ (homogeneous), were treated with anhydrous hydrogen iodide as described for 2-iodooctane.¹⁰ Yield, 31 gm.; b.p. 98–99°, 12 mm.; $d_4^{25} = 1.2834$ (*in vacuo*); $n_D^{25} = 1.4872$.

$C_8H_{19}I$. Calculated, C 42.53, H 7.54; found, C 42.72, H 7.26
 $[\alpha]_D^{25} = -1.72^\circ$; $[M]_D^{25} = -4.37^\circ$ (homogeneous)

Levo-4-Azidononane—25 gm. of 4-iodononane, $[\alpha]_D^{25} = -1.72^\circ$ (homogeneous), were added to a solution of 12 gm. of sodium azide (Kahlbaum) in 30 cc. of water and 475 cc. of methanol. The

⁹ Levene, P. A., Rothen, A., and Kuna, M., *J. Biol. Chem.*, **120**, 777 (1937).

¹⁰ Levene, P. A., and Rothen, A., *J. Biol. Chem.*, **115**, 415 (1936).

resulting solution was placed in two pressure bottles and heated at 60° for 4 hours. The azide was isolated as usual.¹⁰ Yield, 13 gm.; b.p. 100°, 23 mm.; $n_D^{25} = 1.4368$; $d_4^{25} = 0.85$ (approximately).

$C_9H_{19}N_3$. Calculated, C 63.86, H 11.31; found, C 63.77, H 11.21
 $[\alpha]_D^{25} = -0.1^\circ$; $[M]_D^{25} = -0.2^\circ$ (homogeneous)

*Dextro-4-aminononane*²—12 gm. of 4-azidononane, $[\alpha]_D^{25} = -0.1^\circ$ (homogeneous), were dissolved in 50 cc. of methanol and 0.3 gm. of Adams catalyst was added. The mixture was shaken with hydrogen at a pressure of 3 atmospheres for 3 hours. The free amine was isolated as usual. Yield, 5 gm.; b.p. 113–114°, 82 mm.; $d_4^{25} = 0.7772$ (*in vacuo*).

$C_9H_{21}N$. Calculated, C 75.44, H 14.78; found, C 75.37, H 14.73
 $[\alpha]_{54.61}^{25} = +0.52^\circ$; $[M]_{54.61}^{25} = +0.75^\circ$ (homogeneous)
 $[\alpha]_{54.1}^{25} = -0.94^\circ$, $[M]_{54.61}^{25} = -1.69^\circ$ (hydrochloride 19% in H_2O)

ON CHONDROSIN

By P. A. LEVENE*

(From the Laboratories of The Rockefeller Institute for Medical Research)

(Received for publication, February 20, 1941)

Chondrosin, a nitrogenous aldobionic acid, is the carbohydrate moiety of chondroitinsulfuric acid. A polymer of N-acetylchondroitinsulfuric acid constitutes the molecule of the latter. In 1913 Levene and La Forge tentatively expressed the structural formula of the acid as a dimer.¹ In 1937 von Fürth and Bruno advanced further evidence towards the dimeric structure of the molecule.² Nevertheless, the question of the molecular size of the substance is in need of further study. The present author's view on this subject has already been expressed by Tipson and Stiller.³

The present day information on the structure of chondrosin is expressed by structural Formula I arrived at by Levene and La Forge in 1913.

In this expression the cyclic structures of the two components are arbitrary, as is also the place of union of the two components. It was the aim of the present investigation to obtain the missing information.

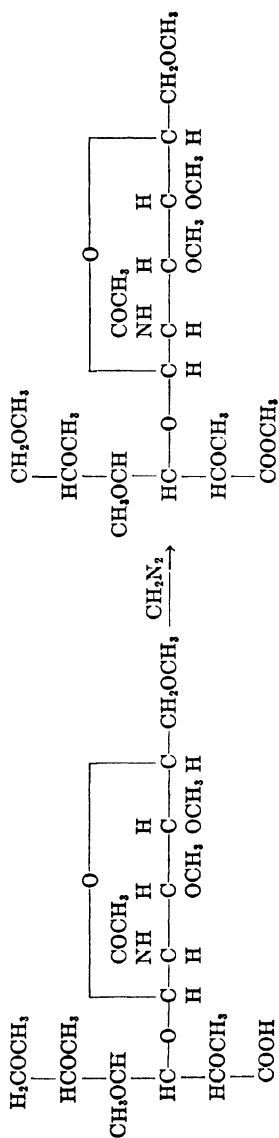
For the elucidation of the position of chondrosamine residue on that of glucuronic acid, it was necessary to eliminate the cyclic structure of the glucuronic acid moiety so that on exhaustive methylation with subsequent hydrolysis this fragment would contain a single free hydroxyl group which could be considered as the one uniting the two components of chondrosin. The chondrosin

* Died September 6, 1940.

¹ Levene, P. A., and La Forge, F. B., *J. Biol. Chem.*, **15**, 155 (1913).

² von Fürth, O., and Bruno, T., *Biochem. Z.*, **294**, 153 (1937).

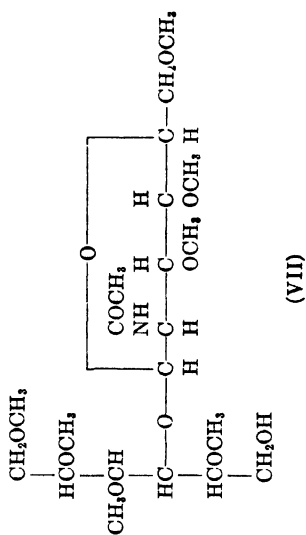
³ Tipson, R. S., and Stiller, E., in Harrow, B., and Sherwin, C. P., *Textbook of biochemistry*, Philadelphia, 105 (1935).



(V)

(VI)

↓
Copper chromite catalyst



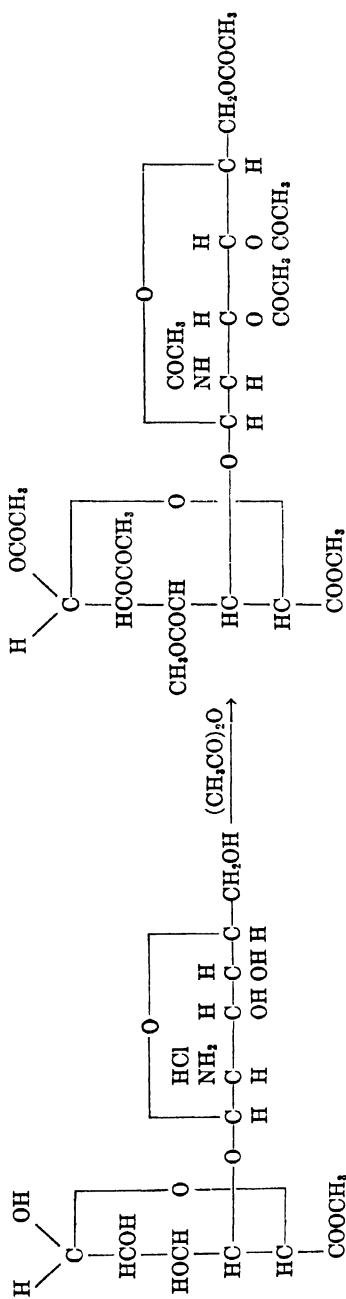
(VII)

was therefore reduced to *l*-gulonochondrosaminide. However, inasmuch as chondrosin could not be obtained in crystalline form--and therefore there was no absolute certainty of its purity--efforts were directed towards the preparation of a crystalline derivative. Such a derivative was found in the chondrosin methyl ester hydrochloride (II) which served as starting material for further study. The methyl chondrosaminido-*l*-gulonate (III) could not be crystallized, but its octaacetate (IV) was obtained in crystalline form. The yield of the crystalline acetate never exceeded 45 per cent of the theory and the average yield was about 40 per cent. This acetate was methylated by the methyl sulfate method, yielding a not completely methylated derivative of N-acetylchondrosaminidogulonic acid which was esterified and exhaustively methylated by the Purdie method. The product (VI) was obtained in crystalline form, although again the yield was not large. For further work it was considered advantageous to convert the methylated gulonic acid residue into the corresponding sorbitol for the reason that partially methylated gulonic acid derivatives are not known, whereas partially methylated sorbitols are either known or quite accessible. Product VI was therefore reduced catalytically to the N-acetyltrimethylchondrosaminide of tetra-O-methylsorbitol (VII) which also was obtained in crystalline form.

The individual steps are summarized in Formulas I to VII.

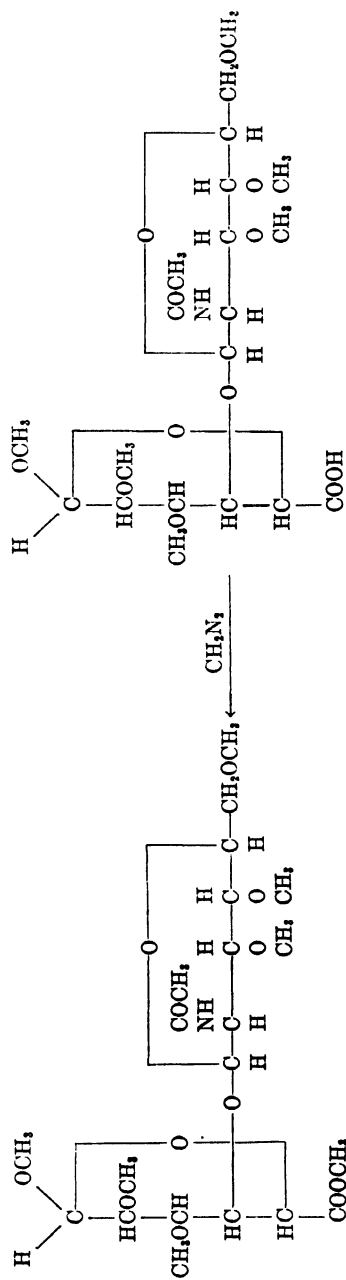
Although it was considered advantageous to prepare the sorbitol derivative for the purpose of elucidating the place of union of the two components of chondrosin, yet it was realized that when the task was to isolate the methylated chondrosamine moiety the completely methylated chondrosin would offer advantages inasmuch as it might permit the separation of the two components obtained on hydrolysis, advantage being taken of the acid properties of the glucuronic acid derivative.

Again the crystalline ester hydrochloride of chondrosin served as starting material. The acetate derived from it was in part crystalline, in part amorphous. It was found, however, that both fractions on methylation gave practically the same exhaustively methylated product which, however, was not obtained in crystalline form. The set of reactions leading to the methyl ester of N-acetylhexamethylchondrosin (XI) is given in Formulas VIII to XI.



(IX)

CH:



(X)

(IX)

The most favorable conditions for the hydrolysis of either the methylated sorbitol or the methylated glucuronic acid derivatives have not yet been found.

EXPERIMENTAL⁴

Preparation of Chondroitinsulfuric Acid—For the preparation of larger quantities of the material required for this investigation, the procedure earlier developed in this laboratory was followed. The crude material was obtained as the lead salt. The wet salt was heated on a water bath and glacial acetic acid was added until complete solution occurred. The mixture was then cooled and more glacial acetic acid was added; this reprecipitated the salt. The operation was repeated as long as the lead salt could be redissolved in glacial acetic acid; as its purity increased the salt became more difficult to redissolve. When redissolution could not be repeated, the salt was washed in glacial acetic acid until the washings became colorless. The lead salt was then washed with alcohol until all adhering acetic acid was removed. The lead salt was converted into the barium salt as previously described. After three precipitations, the barium salt can be used for the preparation of chondrosin. When purer material is desired, the barium salt is dissolved in a minimum volume of a hot 50 per cent solution of $\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$ and precipitated with an equal volume of 95 per cent alcohol. The product is redissolved in water and reprecipitated with an equal volume of alcohol until an aqueous solution of the barium salt is free from chlorine ions.

$\text{C}_{28}\text{H}_{40}\text{O}_{22}\text{N}_2\text{S}_2\text{Ba}_2$. Calculated. C 27.86, H 3.32, N 2.32, S 5.31, Ba 22.76
Found. " 28.1, " 3.65, " 2.31, " 4.90, " 22.6

Chondrosin Hydrochloride (I)—The earlier procedure for the preparation of this substance was slightly modified. 50 gm. lots of the barium salt were taken up in 150 cc. of 10 per cent hydrochloric acid and heated on the boiling water bath for 1 hour. The traces of barium ions remaining in the filtrate from the barium sulfate were removed quantitatively and the filtrate concentrated below 40° under reduced pressure to a very thick syrup. The

⁴ I wish to acknowledge the assistance of Joseph Lender who for the past 25 years has been my laboratory technician. Without his skill and devotion much of this work would not have been accomplished.

residue was dissolved in a small volume of methanol. Ethanol (99.5 per cent) was then added until no more precipitate formed and precipitation was completed by adding about 1200 cc. of dry ether. The precipitate was redissolved in methanol and reprecipitated with ethanol and ether.

Chondrosin Methyl Ester Hydrochloride (II) and (VIII)—10 gm. of chondrosin hydrochloride were heated under a reflux in a 1 per cent solution of HCl in a mixture of 1 part methanol and 4 parts ethanol. The suspended chondrosin hydrochloride was completely dissolved after the first 2 or 3 hours. At the end of the 24 hours, the solution was concentrated under diminished pressure (generally to 30 to 50 cc.) until a white gelatinous precipitate began to form. This precipitate was redissolved by warming and the solution allowed to stand in the cold until the product had separated as long curved needles; these were recrystallized from a small amount of 99.5 per cent ethanol at 35°, when large balls of fine needles formed. The product had an $[\alpha]_D^{30} = +39.2^\circ$ (7.1 per cent in methanol).

When specially purified chondrosin was used for esterification, the ester hydrochloride separated as long needles in feather form aggregates. For recrystallization it was dissolved in a minimum of boiling methanol, ether was added dropwise, and the mixture allowed to stand. The product then crystallized in elongated platelets; m.p. 165–170° (polarizing microscope).

| | | | | | | |
|---------------------------|-------------|----------|---------|---------|----------|--------------|
| $C_{13}H_{24}O_{11}NCl$. | Calculated. | C 38.48, | H 5.96, | N 3.45, | Cl 8.74, | OCH_3 7.65 |
| | Found. | " 38.6, | " 6.25, | " 3.45, | " 8.53, | " 7.76 |

Methyl d-Chondrosaminido-l-Gulonate (III)—10 gm. lots of the chondrosin methyl ester hydrochloride in 150 cc. of methanol were reduced over Raney catalyst in an atmosphere of hydrogen by heating for 48 hours at 75° under an initial pressure of 2500 and final pressure of 3600 pounds per sq. inch. The catalyst was removed by centrifugation and the filtrate was concentrated to dryness under reduced pressure. The residue was further dehydrated by taking it up first in ethanol and subsequently in benzene and by removing the solvent by distillation under reduced pressure. The operation was repeated several times. Only material showing a negligible reduction of Fehling's solution was employed. The yield from 10.0 gm. was from 8 to 9 gm. The material did not

crystallize. It is soluble in water and methanol and in no other organic solvent to an appreciable degree. It was used for acetylation without further purification or analysis inasmuch as only the crystalline acetate was used for further work.

Heptaacetate of Methyl d-N-Acetylchondrosaminido-l-Gulonate (IV)—A mixture of 5 parts of dry pyridine and 5 of acetic anhydride was cooled to -40° , and introduced into the flask containing 1 part of the material (III) to be acetylated. After occasional shaking the mixture was allowed to stand overnight. The reaction product was treated in the usual way. The chloroform extract was concentrated under reduced pressure. It was then dehydrated by dissolving the residue in ethanol and benzene and evaporating the solution to dryness under reduced pressure, the operation being repeated several times. The final residue was taken up in a minimum of methanol; dry ether was added in small portions until, on scratching, crystals began to settle out. Crystallization was allowed to continue overnight. The mother liquor was again dehydrated and again taken up in ethanol and ether and often a second crop of crystals was obtained. The yield from 8 gm. of reduced material was 4 to 6 gm. of colorless prismatic needles; m.p. 122° .



Calculated. C 49.22, H 5.84, N 1.98, OCH_3 , 4.39, COCH_3 , 48.66

Found. " 49.2, " 5.94, " 2.07, " 4.11, " 48.6

$[\alpha]_D^{25} = -21.3^{\circ}$ (3.2% in absolute ethanol)

Heptamethyl Ether of Methyl d-N-Acetylchondrosaminido-l-Gulonate (VI)—To 5 gm. of the crystalline acetate (IV) dissolved in 30 cc. of methanol in the methylating flask,⁵ 60 cc. of carbon tetrachloride, 50 cc. of methyl sulfate, and 10 cc. of water were added. The whole was emulsified by vigorous stirring, and 50 cc. of 42.2 per cent sodium hydroxide were added at the rate of a drop a second. The stirring was continued half an hour, with the bath at 50° . The temperature of the bath was then raised to 60° ; 50 cc. of methyl sulfate were added rapidly, and 50 cc. of alkali at the rate of 2 drops per second. This was repeated until in all 350 cc. of methyl sulfate and 400 cc. of the alkali had been added. The

⁵ Levene, P. A., and Kuna, M., *J. Biol. Chem.*, **127**, 49 (1939).

entire operation lasted 3.5 to 4 hours. The temperature of the bath was then raised to 75° and stirring was continued for an additional 3 hours. The reaction product was cooled, neutralized to initial bluing of Congo paper, and extracted with chloroform. The dry extract was concentrated, dissolved in benzene, and again concentrated. The operation was repeated several times and finally several times in a similar manner with ether. The yield from 5.0 gm. was about 2.0 gm. The product (V) was not fully methylated. Approximately 20 gm. of such material were esterified by means of diazomethane and further methylated by the Purdie method, drierite being added with the silver oxide. After two methylations, the methoxyl content was 45.8 per cent. The methylation was repeated. When the reaction product was finally concentrated from a benzene solution, it crystallized in the distilling flask. It was allowed to stand in the refrigerator for several days and finally the crystalline mass was separated from the mother liquors by triturating with a cooled solution of ether and pentane. The amorphous material, which constituted the major part of the reaction product, contained 47.9 per cent of methoxyl (theory 48.5 per cent). The crystalline material was recrystallized by dissolving in a minimum of ether and adding pentane; m.p. 67° (polarizing microscope).



Calculated. C 51.65, H 8.08, N 2.74, COCH₃ 8.41, OCH₃ 48.53

Found. " 51.6, " 8.09, " 2.83, " 8.32, " 48.9

$[\alpha]_D^{25} = -4.8^\circ$ (15% in ethanol)

N-Acetyltrimethylchondrosaminidotetramethylsorbitol (VII) — A mixture of 3.5 gm. of the foregoing compound (VI) in 100 cc. of methanol and 10 gm. of specially prepared copper chromite catalyst was heated for 7 hours at a temperature of 175° and initial pressure of 2800 pounds per sq. inch. The catalyst remained perfectly black. The water-clear filtrate from the catalyst was concentrated nearly to dryness. The residue was dissolved in benzene and concentrated to dryness. This operation was repeated several times. Finally, the product was concentrated to dryness from its solution in ether. After the last operation had been repeated several times, a crystalline mass formed in the distilling

flask. It was recrystallized from a minimum portion of ether by adding pentane, and finally from a minimum of benzene by addition of pentane. Platelets; m.p. 55–57° (polarizing microscope).



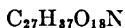
Calculated. C 52.16, H 8.55, N 2.90, COCH₃ 8.90, OCH₃ 44.9

Found. " 52.2, " 8.46, " 2.85, " 8.78, " 44.9

$[\alpha]_D^{25} = -44.2^\circ$ (2.5% in CHCl₃)

Heptaacetylchondrosin Methyl Ester (IX)—20 cc. of acetic anhydride containing 2 gm. of sodium acetate were heated to boiling in a small flask provided with a reflux condenser. The flask was then removed from the flame and 3.0 gm. of chondrosin methyl ester hydrochloride were added. A lively spontaneous reaction followed. The material was refluxed for 6 minutes, then poured on ice, and extracted with chloroform. The chloroform extract was evaporated to a small volume. Toluene was then added and distillation continued until a crystalline deposit formed. This was removed by filtration and the mother liquor was concentrated to dryness. The residue was then dissolved successively in toluene, in benzene, and then in a solution of benzene and ether and finally concentrated to a dry foam. This material on standing in the cold in ethereal solution gave a second crop of the crystalline material. The combined yield from 15 gm. of chondrosin ester hydrochloride was 8 gm. of the crystalline and 16.0 gm. of the amorphous material.

The crystalline material on recrystallization from 99.5 per cent ethanol formed long prismatic needles melting at 100°. After two additional recrystallizations, the crystals softened at 98° and melted at 99–100°.



Calculated. C 48.87, H 5.62, N 2.11, COCH₃ 45.41, OCH₃ 4.68

Found. " 48.8, " 5.67, " 2.08, " 45.2, " 4.7

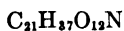
$[\alpha]_D^{25} = +12.2^\circ$ (7.3% in CHCl₃)

N-Acetylhexamethylchondrosin Methyl Ester (XI)—As the crystalline acetate (IX) offered no advantages over the amorphous product, the mixed material was used for methylation.

To 12.0 gm. of the acetate in 25 cc. of methanol and 15 cc. of water, 75 cc. of methyl sulfate were added. The mixture was vigorously stirred, and 100 cc. of 42.2 per cent sodium hydroxide

were then added at the rate of 1 drop in 3 seconds, the temperature of the bath being maintained at 45°. This operation lasted 3.5 hours, at the end of which time the solution gave a barely perceptible reduction test on boiling with Fehling's solution. Stirring at the same temperature was continued for another half hour. The temperature of the bath was then raised to 75°, and 175 cc. of methyl sulfate and 250 cc. of alkali were gradually added. The alkali was added at the rate of at first 1 and later 2 drops per second. The rate of addition of the methyl sulfate was such that it was complete before all the alkali had been added. Stirring was then continued for 40 minutes. Finally, the bath was brought to 100° and stirring was continued for 20 minutes. The yield was 4.0 gm.

This material (X) was dissolved in ether and treated with diazomethane in the usual way. The solution was concentrated to dryness from benzene and again from anhydrous ether. The residue was taken up in ether and precipitated by pentane. After two methylations by Purdie's method, the substance contained 42.3 per cent methoxyl. After two additional methylations, the product (OC_2H_5 , 42.2 per cent) was dissolved in ether, and pentane was added to opalescence. On standing overnight at about -5° , a small quantity of oily material settled out. The supernatant liquid was concentrated to a thick syrup. $n_D^{25} = 1.4702$.



Calculated. C 50.90, H 7.53, N 2.83, COCH_3 8.69, OCH_3 43.84

Found. " 50.7, " 7.75, " 2.79, " 8.64, " 43.9

$[\alpha]_D^{25} = -5.2^\circ$ (6.9° in CHCl_3)

ON THE CARBOHYDRATE GROUP OF EGG PROTEINS. III

By P. A. LEVENE*

(From the Laboratories of The Rockefeller Institute for Medical Research)

(Received for publication, February 20, 1941)

In the first publication on this subject from this laboratory,¹ it was stated that the study was a continuation of the work of Fraenkel and Jellinek and was undertaken at the personal suggestion of Professor Fraenkel. Our experiments at the time improved the method of preparation of the substance and led to the conclusion that the unit of the polysaccharide was a trisaccharide. Since then several publications have appeared dealing with the subject, some confirming and others criticizing our results. Yet there is no evidence that any of the later workers were dealing with purer substances than that prepared by Levene and Mori.¹ The necessity of having a pure substance for the study of the details of its structure is self-evident, particularly when the substance has the properties of a nitrogenous polysaccharide. The difficulties one encounters in using an impure substance are well demonstrated by the recent experience of Stacey and Woolley.² The improvement introduced by us was not only in the use of mercuric sulfate, as stated by Neuberger,³ but also in the following three steps: first, *more exhaustive hydrolysis*, for we have found that on short hydrolysis some peptides still remain attached to the carbohydrate and the nitrogen content of such materials cannot be reduced below 4.5 to 5 per cent. The second very important step is the method of *decomposition of the lead salts*. Only a small part of the lead salts is carbohydrates; most of the other substances

* Died September 6, 1940.

¹ Levene, P. A., and Mori, T., *J. Biol. Chem.*, **84**, 49 (1929).

² Stacey, M., and Woolley, J. M., *J. Chem. Soc.*, 184 (1940).

³ Neuberger, A., *Biochem. J.*, **32**, 1435 (1938).

entering into the basic lead acetate fraction have a higher acid dissociation constant than the carbohydrates. Therefore, that part of the lead precipitate should be utilized which is decomposed by carbon dioxide. This procedure was introduced by Levene and La Forge in 1913. The final step consists in *removing the adhering amino acids* by means of mercuric sulfate.

In 1938 the study of the structure of the polysaccharide was resumed and a quantity of a perfectly white amorphous powder having a nitrogen content of 2.78 per cent and a rotation of $[\alpha]_D^{30} = +35^\circ$ (in 5 per cent HCl) was prepared.

Two ways were considered for the study of the details of the structure of the polysaccharide. One was direct methylation of the polysaccharide; the other was the preparation of a disaccharide from the polysaccharide with the object of reducing the terminal carbonyl group. The hydrolysis of the reduced product should yield only one sugar, the second component being a hexitol. The nature of the former should then indicate the order of union of the two components. If the trisaccharide should be readily cleaved into a disaccharide, it would be admissible to assume that the two mannoses are linked to each other and that the glucosamine occupies a terminal position.

Both methods were given a trial. The polysaccharide (N = 2.78 per cent, ash = 0.3 per cent) was acetylated and then methylated by the methyl sulfate method and subsequently remethylated three times by Purdie's method. A substance was obtained which seemed analytically better than that described recently by Stacey and Woolley.²

Theory for a methylated trisaccharide:

C 52.5, H 8.1, N 2.0, OCH₃ 45.2, COCH₃ 6.3

" 48.1, " 7.8, " 4.3, " 31.5, " 9.7 (Stacey and Woolley)

" 56.6, " 8.1, " 2.9, " 39.6, " 4.9 (Levene)

As the polysaccharide apparently suffered partial oxidation, work was concentrated on the preparation of *di* and *trisaccharides* and a substance having the composition of a disaccharide was obtained. Its N value was 4 per cent and the reducing power 50 per cent calculated as glucose. The end-group of this substance was reduced by means of Rancy catalyst. The product obtained in this manner no longer showed a perceptible reduction of Fehling's solution.

The substance was converted into the acetyl derivative which

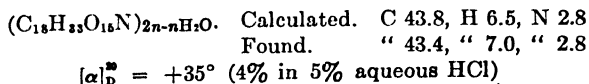
was then hydrolyzed. On hydrolysis of this substance, glucosamine hydrochloride was obtained in a yield comparable to that from the disaccharide.

This observation would then indicate that the reduced substance is mannitolglucosaminide and that the parent disaccharide is *d*-mannoseglucosaminide. When 0.5 gm. of the parent disaccharide and 0.5 gm. of the reduced product respectively were hydrolyzed with 4 per cent hydrochloric acid, the first yielded 0.240 gm. of mannosephenylhydrazone, whereas the second yielded none. It was therefore quite obvious that the second contained no mannose, for whereas the solution of the first product was deeply colored, the second remained practically colorless. Thus it seems quite conclusive that in the disaccharide, the glucosamine residue is bound glycosidically to mannose. The exhaustive methylation of the mannitolglucosaminide with subsequent hydrolysis should then elucidate the place of linkage of the glucosamine residue to the mannose.

There is another point in connection with the structure of the carbohydrate group of egg proteins; namely, the presence of galactose in its molecule. Sørensen⁴ on the basis of colorimetric observation arrived at the conclusion that the carbohydrate contained a galactose residue. The attempts to obtain mucic acid from the products of oxidation of the hydrolysate of the polysaccharide were all negative. In this respect the observations of Neuberger, of Hewitt, and the more recent work of Gurin and Hood⁵ are in harmony with those described here.

EXPERIMENTAL

The carbohydrate was prepared by the previously described procedure. The carbohydrate was a perfectly white powder soluble in water, acids, or alkalies but insoluble in organic solvents. It showed no biuret test and gave a barely colored aqueous solution. The composition of the substance was the following, calculated on an ash-free basis.



⁴ Sørensen, M., *Biochem. Z.*, **269**, 271 (1934).

⁵ Gurin, S., and Hood, D. B., *J. Biol. Chem.*, **131**, 211 (1939).

Disaccharide—5.0 gm. of the polysaccharide were dissolved in 50 cc. of 10 N hydrochloric acid and allowed to stand at room temperature of about 27° for 40 hours. The solution was then diluted to 500 cc. and the Cl ions removed by means of silver acetate. The excess of silver was removed by means of hydrogen sulfide and the filtrate concentrated to dryness. The residue was taken up repeatedly in anhydrous alcohol which was removed by distillation. The final residue was extracted with boiling anhydrous methanol. 2.0 gm. remained insoluble. The mother liquor of this was again concentrated and on second extraction gave a residue of 0.2 gm. Out of the mother liquor by precipitation with ether a third deposit formed.

The 2 gm. fraction was analyzed.

$C_{12}H_{22}O_{10}N$. Calculated. N 4.1, reduction 50% (as glucose)
 Found on ash-free basis. " 4.3, " 47% " "

Reduction with Raney Nickel Catalyst—8.0 gm. of the disaccharide were dissolved in 100 cc. of water. 20.0 gm. of Raney catalyst were added and the mixture was heated for 24 hours under an initial pressure of H_2 of 1600 pounds per sq. inch at 75°, filtered, concentrated nearly to dryness, and taken up in methanol. A granular precipitate formed which remained white on careful drying. The substance was taken up in a minimum of water and reprecipitated with anhydrous alcohol. The substance did not reduce Fehling's solution. It still contained 10 per cent of mineral impurity.

$C_{12}H_{22}O_{10}N$. Calculated, N 4.1; found (ash-free), N 4.1

Acetylation of Mannitolchondrosaminide and Hydrolysis—The object of acetylation was to make available for hydrolysis a sample of the reduced material free of mineral impurities. 2 gm. of the substance were acetylated with acetic anhydride and fused sodium acetate. The reaction product was extracted with chloroform and the solution washed carefully with water. The chloroform extract was concentrated. The residue was dissolved in benzene and the solution concentrated. The operation was repeated until all acetic anhydride and acetic acid were removed. The residue was then taken up in ether and the solution was concentrated. The operation was repeated. The final product which had the con-

sistency of a thick syrup was not homogeneous but was satisfactory for the present purpose.

$C_{11}H_{19}O_{11}N$. Calculated. C 49.6, H 5.80, N 2.06, $COCH_3$ 50.8
Found. " 45.7, " 5.82, " 2.28, " 44.0

Hydrolysis of the Acetate for Glucosamine—2.0 gm. of the above acetate were hydrolyzed by refluxing over a free flame for 6 hours in 25 cc. of 20 per cent hydrochloric acid. The reaction product was diluted to 200 cc. and then concentrated to dryness under reduced pressure at room temperature. As it did not crystallize, the residue was again taken up in 50 cc. of 20 per cent hydrochloric acid and again refluxed over a free flame for 6 hours. The residue on recrystallization from methanol gave 0.054 gm. of glucosamine hydrochloride which was pure after one recrystallization.

$C_6H_{14}O_5NCl$. Calculated, C 33.3, H 6.5; found, C 33.1, H 6.6

Hydrolysis for Mannose 0.5 gm. of the reduced disaccharide was refluxed on a water bath in 50 cc. of 4 per cent hydrochloric acid for 3.5 hours. The solution remained practically colorless. The hydrolysate was neutralized and concentrated under reduced pressure to 6 cc. 0.5 gm. of phenylhydrazine hydrochloride was added. On long standing, oily droplets settled out. The residue was filtered off and was taken up in a minimum of 50 per cent alcohol. A few crystals were seen in the flask, but not enough to weigh.

Control—0.5 gm. of the original disaccharide was hydrolyzed as above. The hydrolysate was brownish in color with a small dark flocculent precipitate which was removed by filtration. The filtrate treated as above yielded 0.240 gm. of phenylhydrazone of mannose. On recrystallization from 50 per cent alcohol it was purely crystalline without amorphous droplets.

Hydrolysis and Oxidation for Mucic Acid—In an earlier experiment (1938 39), 1.0 gm. of the polysaccharide was dissolved in 20 cc. of 5 per cent nitric acid and heated in a sealed tube at 100° overnight. The following morning the tube was opened. The solution to which 20 cc. of nitric acid, specific gravity 1.42, were added was allowed to stand overnight. It was then heated over a free flame for 5 minutes and then concentrated nearly to dryness on a watch-glass. The residue was redissolved in a small portion

of nitric acid and reconcentrated. The operation was repeated until the residue acquired a brittle character. The excess of nitric acid was removed by repeatedly dissolving the residue in water and concentrating the solution to dryness. The residue was dissolved in a minimum of water and allowed to stand in the cold. Mucic acid did not form. By such manipulation 0.200 gm. of galactose yielded 0.100 gm. of mucic acid. A mixture of 0.200 gm. of pure galactose and 0.200 gm. of glucosaminic acid gave 0.110 gm. of mucic acid, and a mixture of 0.200 gm. of galactose, 0.200 gm. of mannose, and 0.200 gm. of chondrosaminic acid gave 0.091 gm. of mucic acid, thus showing that the contamination with other dicarboxylic sugar acids does not affect the precipitation of mucic acid.

After the publication of the paper of Stacey and Woolley, experiments were performed accurately according to their directions but no formation of mucic acid was observed.

THE VERATRINE ALKALOIDS

VIII. FURTHER STUDIES ON THE SELENIUM DEHYDROGENATION OF CEVINE

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In a previous publication (1) we have reported a confirmation at least in part of the results obtained by Blount (2) in the dehydrogenation of cevine with selenium as well as the isolation of several other products also formed during the reaction. The products isolated other than the simpler pyridine derivatives are unique in character and undoubtedly will play an important rôle in the final elucidation of the structure of the veratrine alkaloids. Unfortunately, the yields of these products are rather small because of the complicated course of the decomposition of the alkaloid and they are difficult to isolate owing undoubtedly to the formation of a number of closely related products the properties of which are very similar.

More recently we have turned again to this study with the intention of making a more thorough examination of the reaction mixture in the hope of effecting a more complete separation leading to other products the study of which might aid in the interpretation of the structure of those already isolated and further of increasing the supply needed for chemical investigation. Accordingly we have dehydrogenated a larger amount of cevine for the purpose. The dehydrogenation was carried out as previously reported except that the temperature was raised to 345°.

The products from all of the runs were combined and processed together. The general procedure was to effect a separation first into basic, neutral, and phenolic fractions, after which each fraction was subjected to chromatographic analysis in a chromatograph of the appropriate size prepared from Brockmann's alumina. In

only one case did any of the fractions from the chromatographic separation appear to approach homogeneity, although the method served to give a separation into classes of substances which had approximately the same affinity for the adsorbent and were probably for the most part a mixture of homologues. Each of the fractions coming from the chromatograph was then subjected to microfractional distillation analysis with the use of the apparatus and technique indicated in a previous publication (3). The cevanthridine fraction proved to have too high a boiling point for efficient separation without the danger of pyrolysis in this type of column. Also many of the fractions from the chromatograph yielded less than 0.4 gm. of substance, an amount too small for efficient fractionation.

Fractional distillation served to separate the chromatographic fractions into different molecular sizes and frequently separated more or less completely mixtures of homologues in which the operating conditions were favorable to high efficiency of the column. Usually, however, it was necessary to study further the fractions from the fractional distillation by means of microfractional crystallization as well as by the preparation of derivatives.

This general method of procedure has resulted in the isolation of fifteen degradation products, the formulations of which appear to be reasonably certain, as well as of others in which homogeneity is still in question. The majority of these products have not as yet been reported. The isolation of the following list of substances which includes those already described is therefore reported in this paper: β -picoline C_6H_7N , 2-ethyl-5-methylpyridine $C_8H_{11}N$, base $C_9H_{13}N$, 2-hydroxyethyl-5-methylpyridine $C_8H_{11}ON$, oxyethylmethylpyridine (cyclic ether?) C_8H_9ON , hydrocarbon (4,5-benzohydrindene) $C_{13}H_{12}$, hydrocarbon $C_{17}H_{16}$, hydrocarbon $C_{18}H_{18}$, hydrocarbon $C_{19}H_{20}$, hydrocarbon $C_{24}H_{30}$, cevanthrol $C_{17}H_{16}O$, an oxygen-containing substance $C_{23}H_{24}O$, cevanthridine $C_{25}H_{27}N$,¹ base $C_{20}H_{19}N$, base $C_{26}H_{25}N$.

The relationships of the first five of these substances seem to be quite clear on the basis of the structures already discussed (1). Both the third and fourth substances, which have not been re-

¹ In a succeeding paper, evidence for the formulation of this substance will be presented which seems to favor rather the formula $C_{25}H_{27}N$ than that of $C_{23}H_{25}N$ originally proposed by Blount (2).

ported before from the selenium dehydrogenation, are entirely compatible. Concerning the nature of the remaining degradation products considerable information is now at hand from a study of absorption spectra as well as of certain chemical transformations. This will be discussed in the succeeding papers. As will be seen, the hydrocarbon $C_{18}H_{12}$ has been found to correspond in properties with those of 4,5-benzohydrindene.

EXPERIMENTAL

120 gm. of recrystallized cevine were dehydrogenated in six separate runs as previously described (1) except that the temperature employed was 345° . The volatile material in the ice trap was removed and treated with a slight excess of HCl. The mixture was extracted with ether. After concentration of the ether extract, the resulting residual thick oil (about 1.5 gm.) was investigated by chromatographic analysis followed by fractional distillation in accordance with procedures essentially the same as those used with the major reaction mixture as described below. All that could be obtained from such non-basic material carried over into the ice trap was a series of oils. From these again the only crystalline material that could be isolated appeared to be the $C_{18}H_{12}$ hydrocarbon previously reported. Although the amount of material available was too meager for exhaustive recrystallization, the micro melting point finally reached $104-107^{\circ}$ and showed no depression with a more extensively recrystallized sample of other origin which melted at $116-118^{\circ}$.

$C_{18}H_{12}$. Calculated, C 92.24, H 7.77; found, C 92.12, H 7.65

Analytical data indicated the various oily fractions to be cyclic hydrocarbons in a partial state of dehydrogenation.

The HCl solution which remained after the above ether extraction was treated with excess solid KOH and the basic material which separated was extracted with benzene. The benzene extract was dried over anhydrous K_2CO_3 and run through a chromatograph prepared with benzene and 400 gm. of Brockmann's alumina. As soon as material other than solvent appeared, a volume of 400 cc. was collected. Further fractionation of the material in the chromatograph is described below. This was fractionated first through a Vigreux column to remove most of

the benzene. The residual oil was then placed in a small column having a separating power of roughly ten theoretical plates as measured by mixtures of carbon tetrachloride-benzene. After the residual benzene had distilled, a fraction boiling at 69–75° and under 23 mm. pressure was collected. The volume of this fraction was approximately 1.5 cc. It was presumably a mixture of the previously reported β -picoline and 2-ethyl-5-methylpyridine, since it had a micro boiling point of 165° at atmospheric pressure and the analytical data proved to be intermediate between the figures calculated for C_6H_7N and $C_8H_{11}N$.

C_6H_7N . Calculated, C 77.37, H 7.58

$C_8H_{11}N$. " " 79.27, " 9.15; found, C 78.64, H 8.62

TABLE I

| Fraction No. | Oil bath temperature | Pressure | Weight | Micro b. p. | Analysis | |
|--------------|-----------------------------------------------------|----------|--------|-------------|---------------|---------------|
| | °C. | mm | mg. | °C | C per cent | H per cent |
| 1 | 106 | 8 | 70 | 187 | 79.61 | 9.17 |
| 2 | 106 | 5 | 70 | 189 | 79.59 | 9.76 |
| 3 | 106 | 2 | 70 | 199 | 77.35 | 9.02 |
| 4 | 110 | 0.6 | 70 | 210 | 73.84 | 7.95 |
| 5 | 150 | 0.1 | 60 | 216 | 78.75 | 9.35 |
| 6 | 200 | 0.1 | 100 | 260 | 77.80 | 9.13 |
| 7 | Approximately 200 mg. of residue removed from still | | | | | |

The next fraction was of more constant boiling point and amounted to approximately 1 cc. It had a micro boiling point of 173° at atmospheric pressure and gave the correct analytical figures for ethylmethylpyridine.

Found. C 79.05, H 9.05

The residue in the still which was less than 1 cc. in volume was too small in amount for the column in use and was therefore transferred to a column of the type described previously (3). This column was 5 cm. in height and had a separating power in the neighborhood of eight theoretical plates. The data for the fractionation are given in Table I.

Each of the fractions was investigated carefully for the presence

of a volatile quinoline derivative which, if it had been formed, should have appeared in one of these fractions. However, we were unable to locate such a derivative and our investigation therefore seems to eliminate the possibility of the production of a simple quinoline derivative. The analytical data also indicate nothing higher in carbon or lower in hydrogen than an alkyl-substituted pyridine or oxypyridine.

The Base $C_9H_{13}N$ —Fraction 2 gave analytical data suggesting a formula $C_9H_{13}N$.

$C_9H_{13}N$. Calculated, C 79.93, H 9.70; found, C 79.59, H 9.76

It gave a picrate that crystallized from alcohol in broad thin leaves which melted at 150–151°.

$C_9H_{13}N \cdot C_6H_3O_7N_3$. Calculated, C 49.43, H 4.43; found, C 49.30, H 4.20

This picrate as well as the free base corresponded with the properties of the pyridine base, $C_9H_{13}N$, previously obtained from the zinc dust distillation of cevine (4). A mixed melting point of the picrates showed no depression. Since in the previous publication it was shown that the base upon oxidation with $KMnO_4$ yielded a pyridinedicarboxylic acid possessing one more CH_2 group than isocinchomeric acid, it is perhaps a homologue of ethylmethylpyridine and has the additional CH_2 group in a position which prevents or retards its oxidation.

The Base C_8H_9ON —Fraction 4 gave analytical figures indicating the presence of an oxygen-containing base, although it was evidently still a mixture. 20 mg. of the base were treated with an equivalent of picric acid and the resulting picrate was crystallized from acetone. 25 mg. of heavy rhombs were obtained which melted at 150–151°. After recrystallization, the melting point was 151–152°. This melting point agreed with that previously reported for the picrate of the base C_8H_9ON (1).

$C_8H_9ON \cdot C_6H_3O_7N_3$. Calculated, C 46.14, H 3.30; found, C 46.31, H 3.31

Isolation of the Base $C_8H_{11}ON$ —After collection of the first 400 cc. of eluent from the chromatograph above a second volume of 500 cc. of benzene was collected. Upon fractionation approximately 0.3 gm. of an oil was obtained which appeared mostly to boil in the region of β -picoline and was not further studied.

A liter of ether was then run through the chromatograph. Similar fractionation of the resulting oil (0.4 gm.) also yielded for the most part fractions approaching the boiling point of β -picoline.

500 cc. of methyl alcohol were then run through the chromatograph. Fractionation of this gave, after removal of the solvent, 2 cc. of water and then 1.3 cc. of a viscous higher boiling oil. The latter fraction was placed in a 22 cm. microfractionating column (3). The data for the fractionation are given in Table II.

After Fraction 3 the micro boiling point was fairly constant and since Fractions 2, 7, and 10 all gave similar analytical data, it appeared likely that they were all the same and consisted of a

TABLE II

| Fraction No. | Bath temperature | Column temperature | Pressure | Weight of fraction | Micro b.p. | Analysis | |
|--------------|------------------|--------------------|----------|--------------------|------------|----------|----------|
| | | | | | | C | H |
| | °C. | °C. | mm. | mg. | °C. | per cent | per cent |
| 1 | 102 | 90 | 5 | 20 | 215 | | |
| 2 | 102 | 90 | 5 | 40 | 225 | 69.82 | 7.78 |
| 3 | 102 | 100 | 5 | 80 | 227 | | |
| 4 | 102 | 100 | 5 | 80 | 229 | | |
| 5 | 102 | 95 | 4 | 80 | 229 | | |
| 6 | 102 | 92 | 4 | 80 | 229 | | |
| 7 | 102 | 92 | 4 | 100 | 229 | 69.93 | 8.24 |
| 8 | 102 | 92 | 4 | 70 | 229 | | |
| 9 | 105 | 92 | 4 | 80 | 229 | | |
| 10 | 110 | 98 | 4 | 80 | 229 | 69.83 | 8.18 |
| 11 | 115 | 105 | 4 | 80 | 229 | | |

substance with the empirical formula of a hydroxyl derivative of ethylmethylpyridine.

$C_8H_{11}ON$. Calculated, C 70.02, H 8.08

The picrate could not be induced to crystallize. It gave no color with ferric chloride and could not be methylated with diazomethane. The substance did not have phenolic properties, since it was extracted with benzene in the earlier isolation from a strong solution of KOH. The hydroxyl group appears therefore to be on the side chain. This is supported by the oxidation of the substance with $KMnO_4$ to isocinchomeric acid as follows:

50 mg. of the base were dissolved in 10 cc. of water and treated

with 350 mg. of KMnO_4 . After heating on the steam bath for 6 hours, the MnO_2 was filtered off. The clear filtrate was treated with normal HCl until just acid to Congo red and then 0.5 cc. in excess was added. After concentration to about 1 cc. and chilling, 25 mg. of needles were collected which melted at $260-261^\circ$, depending somewhat on the rate of heating. A mixed melting point with material obtained from the oxidation of 2-ethyl-5-methylpyridine (5) showed no depression.

$\text{C}_7\text{H}_8\text{O}_4\text{N}$. Calculated, C 50.30, H 2.99; found, C 50.65, H 3.08

Isolation of Hydrocarbons—The residue which remained in the original dehydrogenation flask was ground up and exhaustively extracted with ether. The ether was shaken out with 10 per cent HCl . The lower acid layer contained much solid insoluble hydrochloride and was set aside to be treated as described below for the basic part. The dried ether extract gave approximately 7 gm. of residue which was dissolved in 100 cc. of benzene. This solution was run through a chromatograph prepared with 500 gm. of Brockmann's alumina suspended in benzene. As soon as dissolved material began to appear at the lower end, each 100 cc. quantity of eluent was considered a fraction and was evaporated to dryness and weighed. The weights were as follows: Fraction 1, 1.600 gm., oil; Fraction 2, 0.560 gm., oil; Fraction 3, 0.200 gm., oil; Fraction 4, 0.115 gm., oil; Fraction 5, 0.120 gm., oil; Fraction 6, 0.106 gm., oil; Fraction 7, 0.085 gm., oil; Fraction 8, 0.085 gm., oil; Fraction 9, 0.040 gm., oil; Fraction 10, 0.040 gm., partially crystalline; Fraction 11, 0.030 gm., partially crystalline; Fraction 12, 0.110 gm., partially crystalline (from 500 cc. of eluent).

1 liter of anhydrous ether was then run through the chromatograph. This eluted 200 mg. more of material which could not be induced to crystallize. 400 cc. of methyl alcohol were then employed. Upon evaporation this extract proved to contain 2.7 gm. of material which was oxygen-containing and which contained the cevanthrol fraction. Our investigation of this fraction thus far has yielded only cevanthrol (6), although the major portion of the fraction appears to consist of material giving similar analytical data but to have a lower, indefinite melting point. It is probably composed of substances closely related to cevanthrol and with similar properties.

The above Fractions 10, 11, and 12 eluted with benzene yielded partly crystalline residues which appeared to have similar properties and were therefore combined. After sublimation in a high vacuum and two recrystallizations from ether a small amount of material was finally obtained which gave a micro melting point of 181-187°. This material was insufficient for both recrystallization and analysis. The latter indicated a formula of $C_{23}H_{24}O$.

$C_{23}H_{24}O$. Calculated, C 87.29, H 7.65; found, C 87.21, H 7.77

Fractions 1 and 2 from above were combined and placed in a sublimation apparatus. All was collected which distilled up to

TABLE III

| Fraction No. | Weight | Oil bath temperature | Column temperature | B.p. at 760 mm. | Micro m p | Analysis | |
|--------------|--------|----------------------|--------------------|-----------------|-----------|----------|----------|
| | | | | | | C | H |
| | mg. | °C. | °C. | °C. | °C. | per cent | per cent |
| 1 | 100 | 120 | 70 | 80 | | | |
| 2 | 50 | 160 | 90 | 266 | | 90 15 | 9 27 |
| 3 | 70 | 170 | 108 | 293 | | 90 68 | 8.73 |
| 4 | 60 | 180 | 130 | 320 | | 90 63 | 9 06 |
| 5 | 80 | 185 | 145 | 350 | | 90 16 | 9 21 |
| 6 | 130 | 190 | 160 | | Up to 157 | 91 67 | 8 50 |
| 7 | 120 | 195 | 160 | | " " 150 | 91 75 | 8.34 |
| 8 | 130 | 205 | 165 | | " " 99 | 91.99 | 8.31 |
| 9 | 160 | 210 | 165 | | " " 110 | 91 85 | 8 26 |
| 10 | 120 | 215 | 175 | | " " 178 | 91 70 | 8 55 |
| 11 | 135 | 225 | 195 | | " " 177 | 91 80 | 8.11 |
| 12 | 170 | 235 | 210 | | " " 85 | 91 07 | 8 62 |
| 13 | 135 | 235 | 215 | | " " 102 | 90 74 | 9 32 |

an oil bath temperature of 200° and under a pressure of 0.25 mm. The distillate weighed 1.8 gm. This oil was placed in a 22 cm. column (3). The data for the fractionation which was carried out under a pressure of 0.25 mm. are given in Table III.

Although the analytical data did not shift much from fraction to fraction, a closer examination by fractional recrystallization revealed satisfactory separation into different molecular sizes. The original material was apparently composed of mixtures of homologues in various stages of dehydrogenation.

From the boiling point it is obvious that Fraction 1 (Table III) is benzene. Fractions 2 and 3 both yielded crystalline picrates.

Since the picrates appeared to have the same properties, they were combined. Thus approximately 70 mg. of a picrate were obtained which melted at 90–100° (micro melting point). After recrystallization from alcohol heavy orange needles were obtained which melted at 99–103°. This material gave analytical figures which corresponded to a hydrocarbon of the formula $C_{13}H_{12}$.

$C_{13}H_{12} \cdot C_6H_5O_7N_3$. Calculated, C 57.43, H 3.80; found, C 57.77, H 3.62

45 mg. of the picrate were dissolved in ether and the picric acid was removed with 2 per cent NaOH. The recovered hydrocarbon weighed 21 mg. and formed a colorless oil. It was redistilled under reduced pressure. The colorless distillate had only a faint odor and could not be induced to crystallize.

$C_{13}H_{12}$. Calculated, C 92.86, H 7.20, found, C 92.85, H 6.88

A few mg. of the above picrate were recrystallized from ethyl alcohol. The substance had a micro melting point of 103–105°. After a further recrystallization the stout orange needles melted at 106–107°. Since there was insufficient for further recrystallization, this material was used for the comparison with synthetic 4,5-benzohydrindene kindly placed at our disposal by Professor J. W. Cook of the University of Glasgow. This comparison will be discussed in the following paper.

Fractions 4 and 5 (Table III) failed to give crystalline picrates.

The $C_{17}H_{16}$ Hydrocarbon—Fraction 6 which was almost entirely crystalline was recrystallized from ether. 35 mg. of material were obtained with a micro melting point of 147–157°. A further recrystallization gave 19 mg. of thin leaves which melted at 160–165°.

$C_{17}H_{16}$. Calculated, C 92.68, H 7.32; found, C 92.93, H 7.25

The molecular weight determination was made by the Rast method.

0.592 mg. substance : 6.042 mg. camphor, $\Delta = 16.0^\circ$
Mol. wt. found, 222, calculated, 220.13

After a further recrystallization the melting point was 167–169°.

The picrate dissociated very easily but could be crystallized in orange needles from a concentrated solution in benzene. The

micro melting point was 127–129° but the analytical data indicated a much larger proportion of hydrocarbon than is required by the 1:1 molecular ratio.

This hydrocarbon corresponded to the same material for which the formula $C_{17}H_{16}$ was derived previously (1), although the melting point now obtained was considerably higher obviously owing to greater purity.

Fraction 7 after recrystallization in the same manner as in the case of Fraction 6 gave a similar amount of material that showed no depression in a mixed melting point with that from Fraction 6. It was therefore presumably the same substance.

The $C_{18}H_{18}$ Hydrocarbon—Fraction 9 after recrystallization from ether gave 55 mg. with a micro melting point of 85–110°. Upon recrystallization 30 mg. of thin leaves resulted which melted at 109–114°.

$C_{18}H_{18}$. Calculated, C 92.24, H 7.77; found, C 92.28, H 7.65

A portion of this material after recrystallization from ether showed a micro melting point of 114–116° and was identical with the $C_{18}H_{18}$ hydrocarbon previously reported (1). The picrate of this substance also dissociated very easily and was not suitable for characterization.

Fraction 8 behaved similarly upon recrystallization and showed no depression in mixed melting point with the substance from Fraction 9. However, a mixed melting point with the hydrocarbon from Fraction 6 was 102–136°.

The Hydrocarbon $C_{19}H_{20}$ —Fraction 11 was recrystallized from ether. This yielded 50 mg. with a micro melting point of 140–162°. After recrystallization from ether again 24 mg. of broad glistening leaves were obtained which melted at 173–182°.

$C_{19}H_{20}$. Calculated, C 91.88, H 8.12; found, C 91.77, H 8.27

The molecular weight determination was made by the Rast method.

0.583 mg. substance : 6.020 mg. camphor; $\Delta = 14.0^\circ$

Mol. wt. found, 249; calculated, 248.16

The above material after recrystallization from ether melted at 185–188°.

Fraction 10 behaved like Fraction 11 and a mixed melting point showed no depression. However, this material showed a definite depression when mixed with either that from Fraction 6 or 8.

The Hydrocarbon $C_{24}H_{30}$ —Fraction 12 contained much finely divided selenium, although the other fractions were free from this impurity. Apparently the small amount of residual selenium was fractionated out at this point. After removal of the selenium the hydrocarbons remaining proved to be too much of a mixture for successful separation.

Fraction 13 upon recrystallization from ether gave 50 mg. with a micro melting point of 97–106°. After recrystallization broad thin leaves were obtained which melted at 106–109°.

$C_{24}H_{30}$. Calculated, C 90.51, H 9.50; found, C 90.45, H 9.64

The molecular weight determination was made by the Rast method.

0.913 mg. substance : 9.230 mg. camphor; $\Delta = 11.1^\circ$

Mol. wt. found, 320; calculated, 318.24

A further recrystallization yielded material melting at 108–110°.

The aqueous acid layer which remained after removal of the ether solution from the original reaction mixture contained a considerable amount of insoluble precipitate which proved to be the cevanthridine fraction. The HCl solution which was separated from the solid was found to contain only simpler pyridine bases. The solid material was dissolved in chloroform and shaken out with 10 per cent KOH. After the solution was dried over K_2CO_3 , the solvent was removed *in vacuo*. The residue was dissolved in benzene and the evaporation repeated in order to remove chloroform. The residue which weighed 25 gm. was redissolved in 100 cc. of benzene and run through a chromatograph prepared with 1.5 kilos of Brockmann's alumina suspended in benzene. As soon as material began to emerge with the solvent each succeeding volume of 150 cc. was considered a fraction. Fractions 1 to 5 contained only oils that could not be induced to crystallize but which after distillation in a high vacuum gave analytical results closely approximating the values for cevanthridine. Fractions 6 to 12 were all nearly entirely crystalline and constituted the cevanthridine fraction. This combined fraction weighed 5 gm.

and yielded 1 gm. of pure cevanthridine upon recrystallization. A discussion of the analysis, etc., of this substance will be left to a following paper.

The succeeding chromatograph fractions appeared to yield material of a different character. The materials in Fractions 22, 23 and 24 appeared to be the same and were combined. Upon recrystallization a substance was obtained corresponding in properties with that previously reported (1) and melted at 229–230°. However, the formula $C_{26}H_{25}N$ now appears more probable than that of $C_{25}H_{25}N$ originally proposed.

| | | |
|-------------------|-------------|-------------------------|
| $C_{26}H_{25}N$. | Calculated. | C 88.84, H 7.17, N 3.99 |
| | Found. | " 88.77, " 7.25, " 4.35 |
| | | " 88.87, " 7.02, " 4.13 |
| | | " 89.16, " 7.08, " 3.97 |

Methiodide of the Base $C_{26}H_{25}N$ —20 mg. of the base were treated with 5 cc. of methyl iodide and allowed to stand several hours. The collected product after recrystallization from a large volume of methyl alcohol formed fine needles which melted with decomposition at about 295°, depending somewhat on the rate of heating.

$C_{27}H_{28}NI$. Calculated, C 65.71, H 5.71; found, C 65.75, H 5.77

Little material was contained in Fraction 30 from the chromatograph, so the benzene was replaced by anhydrous ether as eluent. Fractions 1 to 4 obtained with ether did not contain material that would crystallize but Fractions 5 to 10 all showed a tendency to crystallize. They were therefore combined and crystallized from ether. 225 mg. of material were obtained which was recrystallized twice from benzene and then from chloroform and melted at 226–231°. A final recrystallization from chloroform with bone-black gave 92 mg. of broad, thin leaves which melted at 233–235°.

| | | |
|-------------------|-------------|-------------------------|
| $C_{26}H_{25}N$. | Calculated. | C 87.88, H 7.01, N 5.12 |
| | Found. | " 88.06, " 7.25, " 5.15 |

This substance added methyl iodide as in the case of cevanthridine, and the analysis of the methiodide supported the formulation above. It crystallized from methyl iodide in yellow leaves which

decomposed at 285–290°, depending somewhat on the rate of heating.

$C_{21}H_{22}NI$. Calculated, C 60.71, H 5.34; found, C 60.86, H 5.41

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THE VERATRINE ALKALOIDS

IX. THE NATURE OF THE HYDROCARBONS FROM THE DEHYDROGENATION OF CEVINE

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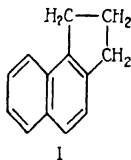
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In the previous paper (1) we have reported the isolation of a series of hydrocarbons from the selenium dehydrogenation of cevine. Since the amount of each substance isolated in a form approaching purity was too small for extended investigation by chemical transformation, our attention has been turned to a study of their absorption spectra. Considerable information is now at hand in the literature regarding the types of absorption spectra in the region of the ultraviolet which are more or less characteristic of the various aromatic ring systems. The formulations of our unknown hydrocarbons as well as their general nature have made them therefore particularly interesting for a study from this standpoint. The results of these investigations are reported in this paper.¹

The simplest hydrocarbon of the series appeared to possess the formula $C_{13}H_{12}$ derived by analysis of the hydrocarbon itself and of its picrate. The number of carbon and hydrogen atoms in this formulation at once places limitations on the possible ring systems which can be considered. A number of combinations of a benzene ring attached to unsaturated 5-membered rings such as indene might be considered but such unsaturated systems could scarcely be expected to withstand the conditions of dehydrogenation. Only a naphthalene ring system with a saturated ring attached can be seriously considered. The possibilities in this

¹ The absorption curves were obtained with a Spekker spectrophotometer and a small Hilger quartz spectrograph. The solvent in each case was absolute alcohol.

category are 4,5-benzohydrindene, 5,6-benzohydrindene, perinaphthane, and one of the four possible methylacenaphthenes. Perinaphthane melts at 60° and gives a picrate melting at 160° (2), whereas 5,6-benzohydrindene melts at 94° and yields a picrate melting at 120° (3). 4,5-Benzohydrindene (4) is an oil and its picrate melts at $109-110^{\circ}$. Our substance was likewise an oil but the small amount of substance available because of the very tedious process involved in its isolation did not permit recrystallization of its picrate to a constant melting point. The melting point, however, on the final recrystallization changed only from $103-105^{\circ}$ to $106-107^{\circ}$. It therefore appeared to approach closely the properties of 4,5-benzohydrindene (Formula I). Further



identity now appears to have been definitely established by a direct comparison with synthetic material which was very kindly placed at our disposal by Professor J. W. Cook of the University of Glasgow. A mixed melting point of the picrates from both sources did not show an appreciable depression and the two substances appeared identical in all their properties. Comparison of the ultraviolet absorption spectrum of synthetic 4,5-benzohydrindene with that of our oil (Fig. 1) with a few minor exceptions showed a close agreement and gave further strong support to the question of identity.

The next hydrocarbon of the series isolated appears from the analysis to possess the formula $C_{17}H_{16}$. Empirically this formulation allows for a total of ten double bonds plus rings and corresponds to a trimethylphenanthrene or an anthracene. However, its ultraviolet absorption spectrum curve is quite different from that of either anthracene or phenanthrene (Fig. 2). The difference appears to be sufficiently great to eliminate the double bond arrangement of either of these ring systems from serious consideration.

The formulation of the next three hydrocarbons, $C_{18}H_{18}$, $C_{19}H_{20}$,

and $C_{24}H_{30}$ respectively, also implies a total of ten double bonds plus rings and this fact, along with their common origin, suggests a close relationship in the ring structure of the four hydrocarbons. The striking similarity of their absorption spectra as shown in Fig. 3 makes this seem even more evident.

Although the type of their absorption spectra seems to be unrelated to either the phenanthrene or the anthracene type, it appears to approach more closely the general type given by naph-

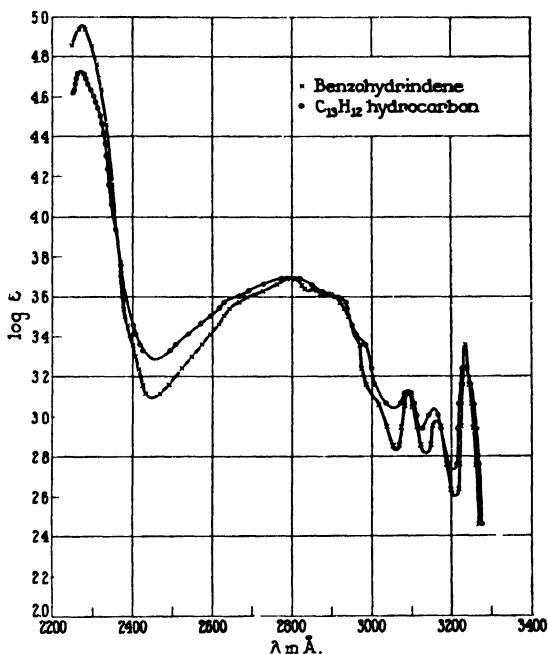


FIG. 1

thalene derivatives. Accordingly, a naphthalene ring system to which are joined three other rings or double bonds might be considered as a possibility. Since a cyclopentenonaphthalene ring system in the hydrocarbon $C_{13}H_{12}$ has been isolated from the same dehydrogenation mixture, it could be suggested that these hydrocarbons contain such a ring system to which either two additional rings are attached or one ring containing a double bond. Of these two possibilities the latter might appear to be more definitely

suggested by the absorption spectra on the naphthalene basis. These show a greater absorption coefficient in general than do naphthalene derivatives which do not carry a group containing a conjugated double bond.

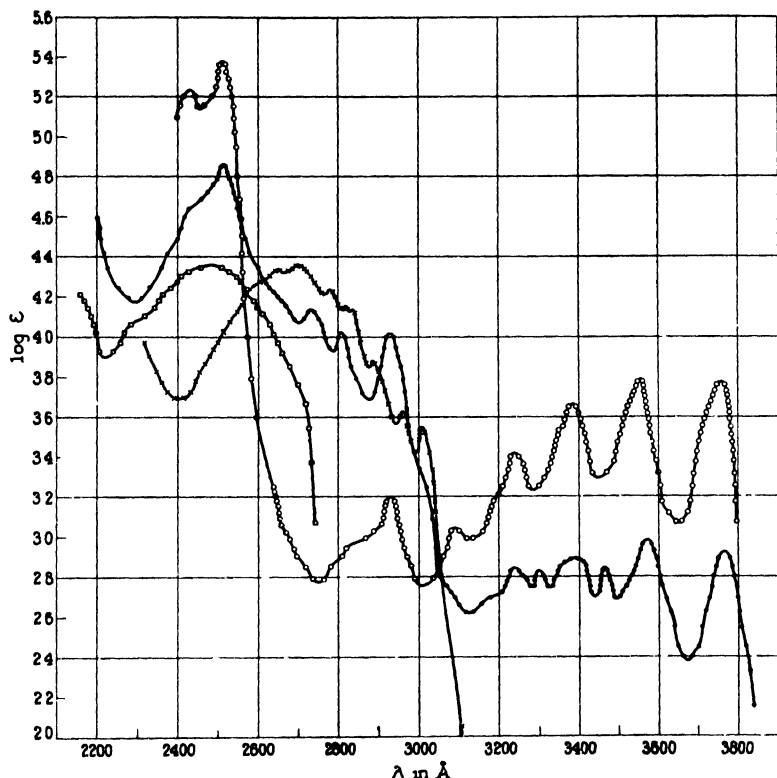


FIG. 2. \times = $C_{17}H_{16}$ hydrocarbon; \bullet = phenanthrene; \circ = anthracene; \square = diphenyl.

It is improbable from general experience that such an extra unsaturated ring could be 5-membered. Should an extra 6-membered ring be attached to the naphthalene ring system in any positions other than the 1,8 or peri positions, the hydrocarbon would then be a tetrahydroanthracene or phenanthrene derivative and under the conditions used for the dehydrogenation from which it was isolated, such a derivative might be expected to be dehydro-

genated to its parent phenanthrene or anthracene. While ordinarily failure to isolate such a derivative from a very complex mixture cannot be considered as final evidence against its presence there, it should be pointed out that a very thorough search was made which had resulted in the isolation of some fifteen substances among which are five hydrocarbons having almost the identical properties which such derivatives would possess. Nevertheless,

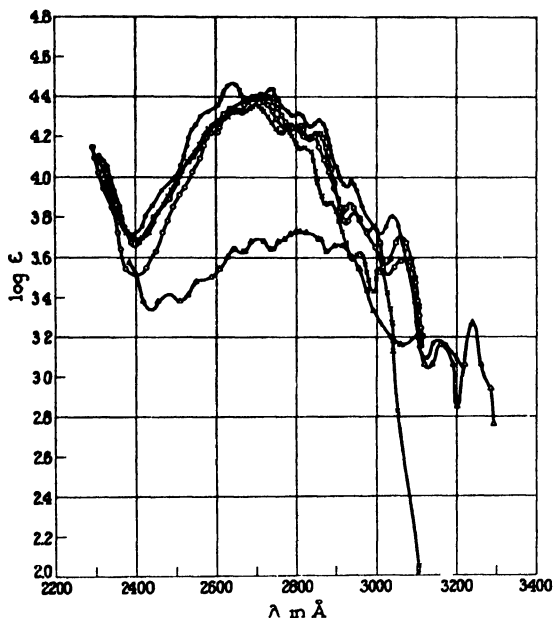
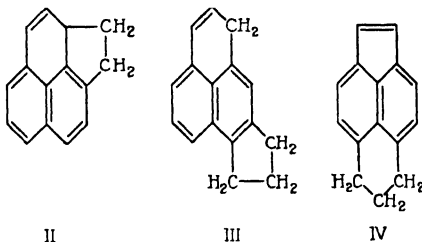


FIG. 3. Δ = $C_{13}H_{12}$; \times = $C_{17}H_{16}$; \circ = $C_{18}H_{18}$, \bullet = $C_{19}H_{20}$; \square = $C_{24}H_{20}$

all attempts to isolate tetrahydrophenanthrenes or anthracenes have failed.

This evidence should be considered together with the fact that there may be present in the alkaloid a hydrogenated naphthalene ring system which is substituted in the 1,8 positions, if our deductions (5) regarding the general structure of decevinic acid and the assumption of its primary character are correct. Thus the possibility must be considered that the more complex hydrocarbons may contain the cyclopentenonaphthalene ring system to which a further ring is joined at the peri positions. In accordance with

this, there may be suggested two structures which are derivatives of the little studied benzonaphthene ring system, Formulas II and III, and a third structure, Formula IV. In each case a number of modifications are of course conceivable on the basis of different arrangements of the double bonds.



From the work of Pestemer and Manchén (6) the approximate effect on the absorption spectrum of a double bond conjugated with the naphthalene ring system can be seen. The absorption coefficient is increased considerably and the bands are shifted toward the longer wave-lengths, an effect in conformity with past experience for the conjugation of a double bond with an aromatic nucleus. The opposite of this last effect has been noted in the case of our substances, if referred to naphthalene.

The question might persist as to whether the reversed shift noted with our substances could be due to any one if not all of the possible arrangements of rings and double bonds in the benzonaphthene ring system.

Since the synthesis of any of the ring structures represented above is a major research in itself and since hydrocarbons with this ring structure appear to be little if at all investigated, it seemed advisable to turn to simpler known substances in order to see what arrangement of double bonds might be indicated in our hydrocarbons by the study of the absorption spectra of such model substances.

Accordingly, the yellow ketone of perinaphthene (benzonaphthene) was prepared according to the directions of Fieser (2) and subjected to dehydrogenation under the same conditions used for cevine (1). A hydrocarbon could be isolated readily which, however, did not give the analytical data expected for perinaphthene but rather those for a hydrocarbon with 2 more H atoms,

viz. perinaphthane, produced presumably by disproportionation. The melting point also agreed with that reported for perinaphthane.

Although such behavior seemed in itself to be against the likelihood of the more complex hydrocarbons discussed above withstanding the dehydrogenating effect of selenium, we have gone further and synthesized a hydrocarbon with the empirical formula of methylperinaphthene by the action of methyl magnesium iodide on perinaphthenone. Although we had expected the initial formation of an alcohol which could then be reduced and dehydrated to methylperinaphthene, a hydrocarbon among other products resulted which gave the proper analytical figures for the desired one. The mechanism of its formation is obscure but may be possibly due to the reducing action of the reagent (7). Aside from the hydrocarbon a yellow solid was also isolated which melted at 87–88° and gave analytical figures corresponding to the empirical formula of $C_{14}H_{10}O$. The exact nature of this product was not determined.

The hydrocarbon melted at 63–65° and proved to be somewhat unstable. Although it could be distilled in a high vacuum to give an almost colorless crystalline solid, upon standing at room temperature a green color developed in the course of a few hours. The absorption spectrum in ethyl alcohol is represented by the curve given in Fig. 1.² Comparison of this curve with the absorption spectra curves of simple naphthalene derivatives shows that in this derivative the conjugated double bond increases the absorption and also displaces the bands considerably toward the longer wave-length. This is a result in agreement with the absorption shown by propenylnaphthalene (6) and therefore cannot very well account for the type of absorption shown in Fig. 3 for the hydrocarbons from cevine.

We have further subjected the hydrocarbon methylperinaphthene to treatment with selenium under the same conditions used for the preparation of the cevine hydrocarbons in order to see whether it would withstand such treatment or possibly undergo

² In a private communication, Dr. Fieser of Harvard University has kindly sent us the absorption spectrum curve of a methylperinaphthene, possibly isomeric with our substance. This curve is in good agreement with that obtained with our substance.

a rearrangement of double bonds to the forms most stable under such conditions. The hydrocarbon isolated in rather poor yield from the resulting mixture crystallized when placed in a freezing mixture but was liquid at room temperature. The analytical data indicated formation of methylperinaphthane by addition of 2 atoms of hydrogen owing to disproportionation and were in agreement with the result of the dehydrogenation of perinaphthenone. While these results are not sufficient to rule out entirely

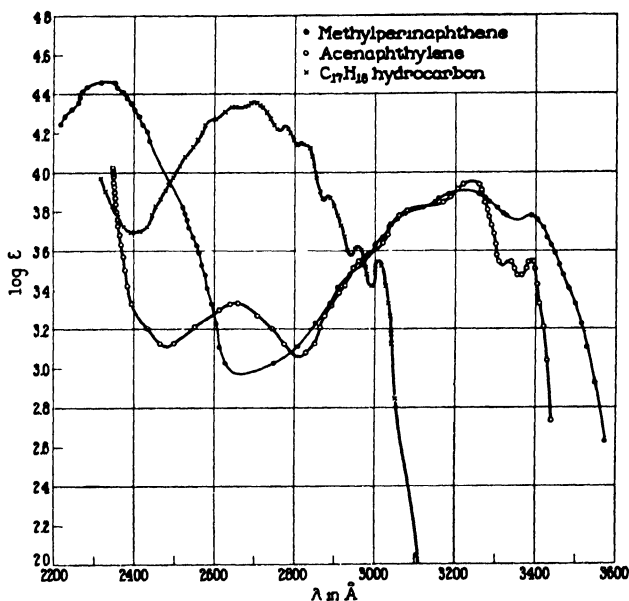


FIG. 4

such general structures as are represented in Formulas II and III, they do serve to make them seem even less likely than other general considerations would indicate.

The formulation represented in Formula IV would be that of an acenaphthylene derivative. Acenaphthene is reported not to dehydrogenate with selenium to acenaphthylene (8) but, nevertheless, the absorption spectrum of the latter was determined and is shown in Fig. 4. This curve seems to be sufficiently different from those of the hydrocarbons from cevine to remove this possibility from consideration.

Of various other known types of absorption spectra, that of diphenyl (Fig. 2) seems to offer considerable similarity to that of our hydrocarbons. The general shape of the curve is approximately the same as well as the intensity of the absorption. The curve of any one of the hydrocarbons, however, covers a region approximately 200 Å. displaced toward the longer wave-lengths from that of diphenyl. This is an effect shown by many examples in the literature to be caused by the substitution of alkyl groups or saturated rings on an aromatic nucleus. If any of the four hydrocarbons is a diphenyl derivative, two further saturated rings must be present in order to make up the ten necessary double bonds or rings and thus conform with the established empirical formulas. Thus two general possibilities remain; *viz.*, a cyclopentenofluorene derivative and the other a cyclopenteno-9,10-dihydrophenanthrene derivative. Both would be expected to give a somewhat modified diphenyl type of absorption curve.

A comparison of the curves of fluorene, 9,10-dihydrophenanthrene, and the $C_{17}H_{16}$ hydrocarbon is shown in Fig. 5. The curves of fluorene and 9,10-dihydrophenanthrene were replotted from data taken from the curves of Askew (9).

Before a 9,10-dihydrophenanthrene derivative can be seriously considered, the possibility that such a derivative would resist dehydrogenation to the corresponding phenanthrene would have to be weighed. That such a possibility cannot be entirely dismissed may be derived from the experience of Bergmann and Weizmann (10) who found that 1,2-dimethyl-3,4,9,10,11,12-hexahydrophenanthrene as well as its 7-methoxy derivative did not dehydrogenate readily to the corresponding phenanthrene and only in poor yield upon long treatment. Oils giving the proper analytical data for dihydro derivatives were isolated which were considered by them to be 9,10-dihydrophenanthrene derivatives. This question, however, must be explored more carefully at a future time.

Thus, the possibility must be considered that the hydrocarbons from the selenium dehydrogenation of cevine could be derivatives of a cyclopentenophenanthrene and if so, barring obscure rearrangements, that cevine could contain such a completely hydrogenated ring structure. However, the failure to dehydrogenate to a phenanthrene has not been encountered in the sterols heretofore as far as we are aware.

The other possibility, a cyclopentenofluorene derivative, would not be an unlikely product of a selenium dehydrogenation. In favor of such a structure can be mentioned the behavior of all

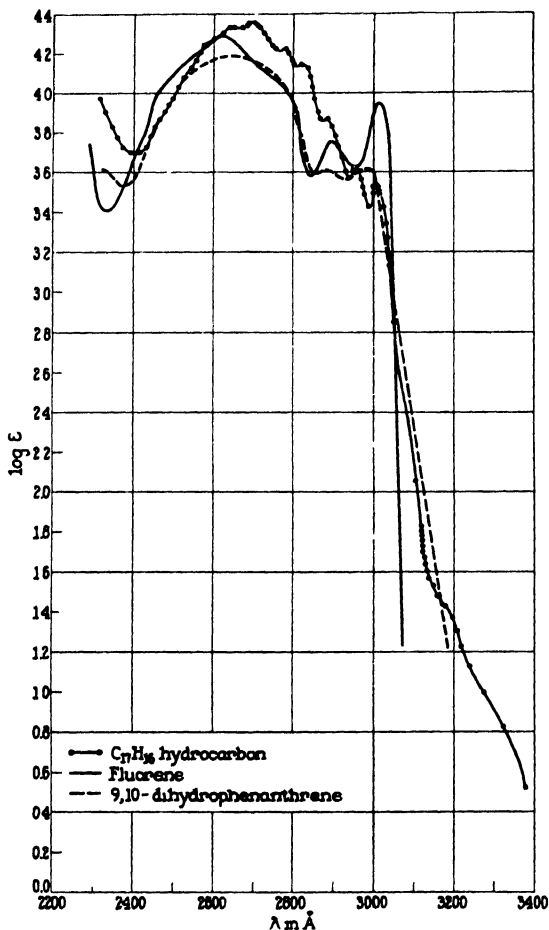


FIG. 5

four of our hydrocarbons when subjected to the Vanscheidt color test (11). When a small amount of the hydrocarbon is dissolved in pyridine, a drop of alcoholic KOH added, and the solution heated with shaking, a yellow color develops which turns gradually

green. The production of color under these conditions has been used to detect the presence of fluorenes, indenes, and derivatives

R
H

having a $C=C-C-C=C$ grouping in hydrocarbon mixtures.

As a check on the selectivity of this test, the behavior of 9,10-dihydrophenanthrene was studied and, as was to be expected, gave a negative test. The 9,10-dihydrophenanthrene was kindly placed at our disposal by Dr. Erich Mosettig.

Should these hydrocarbons prove to be fluorene derivatives and their primary character assumed, then cevine could contain a completely hydrogenated 3,4- or 1,2-cyclopentenofluorene ring system and in the latter case would be somewhat suggestive of the sterol ring system with Ring B 5-membered. However, in either case, the formation of 4,5-benzohydrindene as well as that of the naphthalic acid anhydride obtained from decevinic acid (5) by sulfur dehydrogenation could be explained only by ring enlargement of Ring 2. Both these derivatives were obtained by dehydrogenation, although under temperature conditions much lower than those usually supposed to cause ring enlargement of a cyclopenteno derivative (8). Ring 4 would be attached either as it is represented in the formula, to positions 1,2 on the fluorene nucleus or to positions 3,4 in order to permit the formation of the 4,5-benzohydrindene.

These considerations can only be of the most tentative character based on the observations which we have thus far been able to make. Modification may well become necessary as more data are obtained but our observations serve the purpose of suggesting possible further modes of approach in a field very confusing and experimentally very difficult. There is naturally a temptation to attempt to reconcile the data obtained with something approaching a sterol ring structure, since there are alkaloids already known such as the *Solanum* alkaloids which are considered to have such a structure. However, we have always been confronted with the unique and striking fact that while the hydrocarbon dehydrogenation products of cevine thus far isolated can be shown to have a relation with each other, they do not, to our knowledge, coincide with any sterol dehydrogenation product thus far isolated. Either some unique structural arrangement is responsible for such a

behavior, or, what is more probable, cevine does not bear a close relationship to the sterols.

EXPERIMENTAL

Dehydrogenation of Perinaphthenone with Selenium—1.5 gm. of perinaphthenone prepared according to the directions of Fieser and Hershberg (2) were ground with 4 gm. of selenium and heated in the customary apparatus for dehydrogenation at a temperature of 340° and in an atmosphere of nitrogen for 2 hours. The material remaining in the flask was pulverized and extracted with ether. After evaporation of the ether extract, a residue of 0.45 gm. remained. This was dissolved in 10 cc. of benzene and passed through a chromatograph prepared with 40 gm. of Brockmann's alumina in benzene. The first 100 cc. of benzene coming through the column after the solvent originally present had passed were concentrated through a Vigreux column until a syrup remained. This syrup was then placed in a small still (5 cm. column) of the type previously reported (12) and fractionated. 50 mg. of material distilled at a bath temperature of 140° and under 2 to 3 mm. pressure. This material crystallized in the condenser and melted at 60–63°. After recrystallization from ether-petroleum ether 25 mg. were obtained which melted at 62–63°. The melting point reported by Fieser and Hershberg for perinaphthene was 65.1–65.4°.

$C_{18}H_{12}$ Calculated, C 92.80, H 7.20, found, C 92.11, H 7.04

Since the analytical data indicated impurity, the picrate was prepared and crystallized from acetone. The orange needles had a micro melting point of 148–150°. Fieser and Hershberg (2) report a melting point of 150–151°.

$C_{18}H_{16}O_7N_8$. Calculated, C 57.43, H 3.80, found, C 57.26, H 3.51

Addition of CH_3MgI to Perinaphthenone (Methylperinaphthene) — CH_3MgI was prepared in the customary way with 4 cc. of methyl iodide and a small excess of Mg. To this reagent was slowly added a solution of 2 gm. of perinaphthenone dissolved in a few cc. of benzene diluted with several volumes of ether. There was little evidence of reaction except that the color of the solution became reddish brown. After being refluxed for an hour the mixture

was poured on crushed ice and then acidified with HCl. The ether layer was dried over K_2CO_3 and evaporated to dryness in a sublimation apparatus. 1.6 gm. of material sublimed up to a temperature of 120° and under 0.2 mm. pressure. It was light yellow at first but soon turned reddish brown.

The sublimate was dissolved in 10 cc. of benzene and passed through a chromatograph of 60 gm of Brockmann's alumina in benzene. As soon as material appeared at the lower end, 30 cc. were collected and evaporated. This contained 0.8 gm. of an almost colorless oil which was set aside as Fraction 1. The next 100 cc. of eluent appeared to have a more distinct color and on evaporation yielded 500 mg. of residue which crystallized readily. On recrystallization from ether 270 mg. of stout yellow-green columns were obtained which melted at $87-88^\circ$. Since the analysis showed the substance to be oxygen-containing, it was not investigated further.

$C_{14}H_{10}O$. Calculated, C 86.55, H 5.15; found, C 86.58, H 5.19

Distillation of Fraction 1 and analysis of the distillate indicated it to contain considerable amounts of an oxygen-containing substance. It was dissolved in 10 cc. of commercial isohexane and chromatographed with 60 gm. of Brockmann's alumina in isohexane. The first volume of 50 cc. containing dissolved material was collected. This yielded 300 mg. of an oil which crystallized. It distilled completely at an oil bath temperature of about 130° and 0.2 mm. The substance showed a micro melting point of $60-65^\circ$ but retained a slight green color.

$C_{14}H_{12}$. Calculated, C 93.33, H 6.67; found, C 93.46, H 6.67

If the crystalline material from the chromatograph was recrystallized directly from isohexane without distillation, the substance obtained was practically colorless and had a micro melting point of $63-65^\circ$. However, on standing at room temperature for several hours, color appeared and in a few days the substance changed to a dark green oil.

The absorption spectrum curve of this substance is given in Fig. 4.

Treatment of Methylperinaphthene with Selenium—0.5 gm. of the hydrocarbon was ground with 2 gm. of selenium and dehydro-

generated in the customary vessel in an atmosphere of nitrogen by heating for 1 hour at 340°. The pulverized reaction mixture on extraction with ether yielded an oil which was fractionated in a microdistillation apparatus. 75 mg. of colorless oil distilled up to 140° and under 1 mm. pressure. At a slightly higher temperature 50 mg. further distilled but this fraction had a slight color. The first fraction crystallized directly when chilled but melted again at room temperature. It could not be crystallized from a solvent. On standing it remained colorless. Analysis indicated

TABLE I
Fractionation of Acenaphthylene

| Fraction No. | Column temperature (120° bath temperature) | Weight | Micro m.p. |
|--------------|-----------------------------------------------|--------|------------|
| | °C. | mg. | °C. |
| 1 | 90 | 50 | 50- 75 |
| 2 | 90 | 50 | 88- 91 |
| 3 | 90 | 90 | 89- 92 |
| 4 | 90 | 130 | 83- 87 |
| 5 | 90 | 140 | 73 81 |
| 6 | 90 | 80 | 92-108 |
| 7 | 90 | 30 | 75- 87 |
| 8 | 96 | 140 | 81- 87 |
| 9 | 96 | 50 | 89 93 |

addition of 2 atoms of hydrogen and formation of a methylperinaphthane.

$C_{14}H_{14}$. Calculated, C 92.26, H 7.74; found, C 92.37, H 7.80

Acenaphthylene.—This substance was prepared according to Blumenthal (13) by passing 2 gm. of acenaphthene through a hot tube filled with lead oxide. The entire distillate was crystallized in the cold from about 10 cc. of ether. 1 gm. of needles was collected which proved to be unchanged acenaphthene. The concentrated mother liquor was placed in a microfractionation apparatus (12) having a column 22 cm. in length. The fractionation was carried out under 0.2 mm. pressure (Table I). Fraction 3 was taken for analysis.

$C_{12}H_8$. Calculated, C 94.69, H 5.31; found, C 94.64, H 5.49

Upon recrystallization from ether, golden yellow plates were obtained which had a micro melting point of 92-94°. This material was used for the absorption spectra study. The melting point reported in the literature is 92°.

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THE VERATRINE ALKALOIDS

X. THE STRUCTURE OF CEVANTHRIDINE

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In the previous paper (1) a discussion concerning the nature of the hydrocarbons obtained from the selenium dehydrogenation of cevine was presented on the basis of a study of their ultraviolet absorption spectra. While experiments are in progress to obtain further evidence, we have attempted to extend the same sort of study to the degradation product, cevanthridine, which appears to contain all but a few of the carbon atoms originally present in cevine.

Cevanthridine was first isolated by Blount (2) more than 5 years ago but aside from certain deductions drawn from x-ray studies of the crystals (3) in regard to the molecular dimensions nothing further has appeared which has a bearing on the structure of this substance. However, from data now available in this laboratory it is possible to obtain certain additional suggestions regarding its nature and its relationship to the simpler hydrocarbon degradation products reported by us.

The empirical formula $C_{23}H_{25}N$ was proposed by Blount for cevanthridine on the basis of the analysis of the free base and of its methiodide. In the case of the picrate, however, the analytical data seemed to come consistently high in carbon and this substance was therefore assumed by him to contain less than the molecular ratio of picric acid. Such analytical results we have also encountered with the picrate and while our analytical results are in good agreement with those of Blount for the free base, we have consistently obtained higher carbon figures in the analysis of the methiodide. These results offer the suggestion that cevanthridine may be after all a somewhat larger molecule than originally proposed by Blount.

Our analytical figures seem to indicate rather a formulation of $C_{25}H_{27}N$, which is also in agreement with the analytical results reported by Blount on the picrate and the free base. This formulation is further supported by a study of the hydrogenation product resulting from the catalytic hydrogenation of cevanthridine described below, as well as of derivatives of this substance. Since

TABLE I
Cevanthridine and Derivatives

| Substance | Calculated | | | | | | Found | |
|-------------------------------------|-----------------|------|-----------------|------|-----------------|------|-------|------|
| | $C_{27}H_{25}N$ | | $C_{26}H_{27}N$ | | $C_{25}H_{27}N$ | | C | H |
| | C | H | C | H | C | H | | |
| Cevanthridine | 87.56 | 7.99 | 87.48 | 8.26 | 87.92 | 7.97 | 87.70 | 8.10 |
| | | | | | | | 87.59 | 8.24 |
| Methiodide | 63.01 | 6.17 | 63.68 | 6.56 | 64.56 | 6.25 | 61.30 | 6.10 |
| | | | | | | | 64.19 | 6.39 |
| | | | | | | | 62.8 | 6.1* |
| Picrate | 63.95 | 5.18 | 64.49 | 5.41 | 65.24 | 5.30 | 65.46 | 5.16 |
| | | | | | | | 65.65 | 5.31 |
| | | | | | | | 65.1 | 5.4* |
| | | | | | | | 65.2 | 5.4* |
| Dihydro- | 87.01 | 8.57 | 86.96 | 8.82 | 87.41 | 8.52 | 86.93 | 8.81 |
| | | | | | | | 86.84 | 8.89 |
| Tetrahydro- | 86.46 | 9.15 | 86.44 | 9.37 | 86.91 | 9.05 | | |
| Acetyldihydro- | 83.52 | 8.14 | 83.60 | 8.37 | 84.11 | 8.11 | 83.84 | 8.39 |
| | | | | | | | 83.94 | 8.58 |
| Acetyltetrahydro- | 83.06 | 8.65 | 83.13 | 8.86 | 83.68 | 8.59 | | |
| Dihydro- hydrochloride | 78.06 | 7.98 | 78.33 | 8.22 | 79.01 | 7.96 | 78.44 | 8.43 |
| | | | | | | | 78.57 | 8.33 |
| Tetrahydro- hydrochloride | 77.59 | 8.50 | 77.90 | 8.72 | 78.53 | 8.45 | | |
| Dihydro- <i>p</i> -bromobenzoyl- | 71.98 | 6.04 | 72.36 | 6.27 | 72.99 | 6.13 | 72.66 | 6.87 |
| Tetrahydro- <i>p</i> -bromobenzoyl- | 71.72 | 6.42 | 72.08 | 6.64 | 72.70 | 6.48 | | |

* Results taken from the paper of Blount (2).

it is so often difficult to decide on the proper formulation of a substance of this type, Table I, containing the theoretical values for the various formulas together with the analytical results, is given.

The catalytic hydrogenation of cevanthridine has been studied as part of the investigation. When cevanthridine was hydrogenated in glacial acetic acid with the platinum oxide catalyst of

Adams and Shriner, somewhat more than 2 moles of hydrogen were readily absorbed. The hydrogenation product readily crystallized under ether and melted at 158–159°. This *tetrahydro-cevanthridine* contains a nitrogen atom which must now be secondary in character, since it readily yielded an *acetyl derivative* with acetic anhydride. The acetyl derivative did not form a hydrochloride, as contrasted with the parent base which readily yielded a crystalline *hydrochloride*. The analytical data in Table I are all in conformity with a tetrahydro derivative on the basis of the $C_{25}H_{27}N$ formula now proposed by us.

If either the formula assigned to cevanthridine by Blount or one with one CH_2 more is assumed, the hydrogenated derivative could be only a dihydro derivative, since the addition of 4 hydrogen atoms to either of these formulas would result in formulations not supported by our analytical results. On the other hand, if cevanthridine should yield a dihydro derivative which possesses a secondary nitrogen atom, the only probable structure would be that of an acridine derivative in order to make such a transformation possible. It is difficult to see how an acridine derivative could be formed from an octahydropyridocoline or similar ring system (4) such as appears to be present in the cevine molecule without postulating involved degradation followed by synthesis during the selenium dehydrogenation.

A more probable explanation would seem to be that one ring of the assumed bicyclic nitrogen ring system is ruptured to give an intermediate secondary nitrogen in the ring which is joined to the remaining part of the molecule. This ring could then be dehydrogenated with the remainder of the molecule to a quinoline or isoquinoline derivative. Such a derivative would form a tetrahydro derivative under the conditions used for the hydrogenation and the formulation, therefore, of $C_{25}H_{27}N$ for cevanthridine appears to be the most satisfactory. It is not only supported by the analytical results found with the base itself but also with those obtained with the methiodide and the picrate.

A study of the absorption spectrum of cevanthridine¹ has been made in order to learn if a relationship would be found to the type of absorption shown by the hydrocarbons obtained from the same

¹ The absorption spectra curves were very kindly determined by Dr. George I. Lavin of the Rockefeller Institute.

dehydrogenation. While an obvious relationship does not at once appear with cevanthridine itself, as comparison of Curve 1, Fig. 1, with Curve 2 of the $C_{17}H_{16}$ hydrocarbon will show, the absorption spectrum of the tetrahydro derivative, Curve 3, is strikingly similar to that of the hydrocarbon. The conclusion may therefore be drawn that the same ring structure present in the hydrocarbon is probably also present in the tetrahydro derivative of cevanthridine. Further, since the substitution of a nitrogen directly on an aromatic ring can greatly change the absorption spectrum

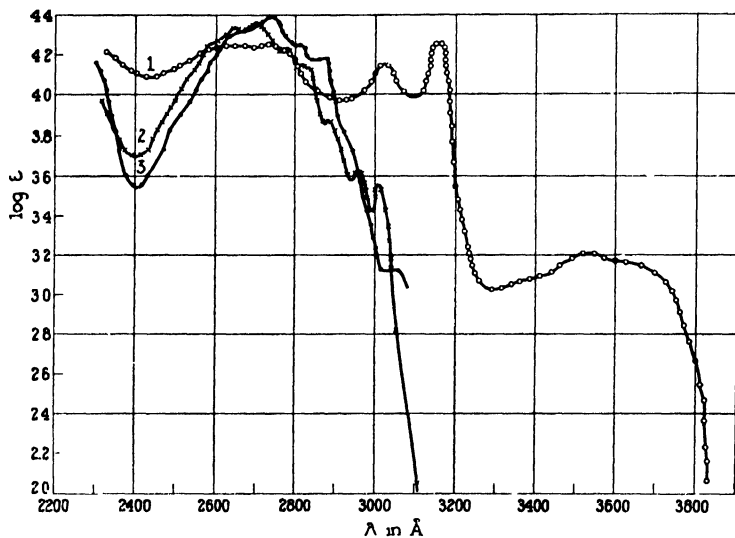


FIG. 1. Absorption spectra of cevanthridine (Curve 1), $C_{17}H_{16}$ hydrocarbon (Curve 2), and tetrahydrocevanthridine (Curve 3).

as demonstrated for example by the difference in the absorptions shown by naphthalene and aminonaphthalene (5), the nitrogen in tetrahydrocevanthridine may be inferred to be at least 1 carbon atom removed from the aromatic nucleus, such as is found in a tetrahydroisoquinoline derivative.

In the previous paper (1), of the three structures discussed as more or less in harmony with the absorption spectra and properties of the hydrocarbons isolated from the dehydrogenation of cevine, a cyclopentenofluorene structure appears perhaps most satisfactory. On such a basis, it is possible that cevanthridine may

have a structure in which an additional 6-membered nitrogen ring is joined at some position on either of the benzene rings and thus may form an isoquinoline derivative. Since alkaloids related to the sterols are known to occur, it might be considered possible that the position of the nitrogen ring could be as shown in Formula I.

But in the attempt to reconcile such possibilities with Ring 2, whether 5- or 6-membered, it is still difficult to see how an octahydropyridocoline or such bicyclic saturated tertiary base could break away intact from two other rings such as Ring 3 or 4 on pyrolysis. It may be that the union between the bicyclic basic portion of the alkaloid and the remainder of the molecule is by means of a single carbon-to-carbon bond and that the formation of the nitrogen ring of cevanthridine on pyrolysis is due to secondary ring closure. In any event much more work will have to be done to correlate properly all of the data at hand.

The empirical formula of cevine is $C_{27}H_{43}O_8N$. This formula when related back to the saturated hydrocarbon without rings implies a total of seven rings plus double bonds. Although originally the possibility had been considered that it might contain a lactone group because of the ability to form a potassium derivative (6), later study has failed to give any evidence of the presence of the necessary carboxyl group. On the other hand, there is a possibility that the presence of an enolic hydroxyl group (7) could account for the formation of a potassium derivative. Catalytic hydrogenation studies (7) have revealed the presence of a single unsaturated linkage and perhaps the one responsible for the enolic hydroxyl (ketone?) group. Absorption spectra studies have failed to reveal absorption bands in the region where benzenoid substances are known to absorb. It would appear, therefore, that there are no double bonds present aside from the one mentioned. In general conformity with this assumption is the failure to isolate anything benzenoid from among the products of the soda lime or zinc dust distillation as well as the number of rings found to be present in the hydrocarbons from the selenium dehydrogenation. Accordingly the ring structure of cevine can be assumed to be hexacyclic.

Four of these six rings may be accounted for by two of the degradation products; namely, the so called octahydropyridocoline fragment and decevinic acid. Together these two derivatives

account for 24 of the 27 carbon atoms originally present in cevine and may be joined to each other in such manner that two further rings are formed. At least one of these rings is very likely 5-membered and appears in the benzohydrindene hydrocarbon. On the basis of the structures postulated for the other hydrocarbons from the selenium dehydrogenation the remaining ring must be 6-membered. Thus all the rings could be accounted for and there would remain the manner in which they are joined to be determined.

EXPERIMENTAL

Cevanthridine Methiodide—30 mg. of cevanthridine when treated with excess methyl iodide at first dissolved but soon deposited crystals. After the mixture was refluxed for several hours, the crystalline material was collected. The melting point was 268–270°, depending somewhat on the rate of heating, and was not changed by recrystallization from acetone. Blount (2) reported 255–256°.

$C_{26}H_{30}NI$. Calculated, C 64.56, H 6.25; found, C 64.30, H 6.10
“ 64.19, “ 6.39

Hydrogenation of Cevanthridine—60 mg. of cevanthridine were hydrogenated in 3 cc. of glacial acetic acid with 50 mg. of platinum oxide catalyst. After the mixture was shaken under approximately 3 atmospheres of hydrogen for 1.3 hours, 2 moles of hydrogen had been absorbed and the absorption became slow. It was interrupted at this point although previous experiments had shown that hydrogenation gradually proceeded further. The filtrate from the catalyst was evaporated to dryness under reduced pressure. After treatment with NaOH solution, the base was extracted with ether. This yielded on concentration 40 mg. of crystalline material which melted at 151–156°. Upon repeated recrystallization a melting point of 158–159° was obtained.

$C_{26}H_{31}N$. Calculated, C 86.91, H 9.05; found, C 86.93, H 8.81
“ 86.84, “ 8.89

When a suspension of cevanthridine in a few cc. of ethyl alcohol containing a slight excess of HCl was hydrogenated, the hydrochloride was directly obtained on concentration. 25 mg. of well

formed crystalline blades were collected. The material did not possess a well defined melting point but sintered and decomposed from 280–295°, depending somewhat on the rate of heating. Neither the analytical data nor the melting point changed upon recrystallization.

$C_{25}H_{31}N \cdot HCl$. Calculated, C 78.53, H 8.45; found, C 78.57, H 8.33
“ 78.44, “ 8.43

Acetyltetrahydrocevanthridine—20 mg. of the tetrahydro derivative were refluxed for 2 hours in acetic anhydride. After concentration the residue was dissolved in ether. The solution was extracted with dilute HCl and dried. On concentration of the ether solution crystalline material appeared. It melted at 202–203° and after repeated recrystallization at 206–207°.

$C_{27}H_{33}ON$. Calculated, C 83.68, H 8.59; found, C 83.84, H 8.39
“ 83.94, “ 8.58

p-Bromobenzoyltetrahydrocevanthridine—Approximately 30 mg. of the tetrahydro derivative were treated with 30 mg. of *p*-bromobenzoyl chloride and 2 cc. of 10 per cent $NaOH$. After the mixture was warmed somewhat to destroy the excess chloride, the product was extracted with ether. The substance separated from ether-petroleum ether and showed a micro melting point of 107–113°.

$C_{32}H_{34}ONBr$. Calculated, C 72.70, H 6.48, found, C 72.66, H 6.87

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THE OXIDATION-REDUCTION EQUILIBRIUM, OVER THE WHOLE pH RANGE, OF OXONINE AND SOME RELATED DYESTUFFS

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It has been one of the major objectives of research in this Laboratory during the past years to prove that the reversibility of any bivalent oxidation-reduction depends on the fact that an intermediate univalent reaction product, a free radical, is capable of existence in sufficient concentration so that this concentration is not a limiting factor for the rate of the over-all process of the bivalent oxidation or reduction. The evidence for such a statement ought to consist in showing that at any point of oxidative or reductive titration at any one pH , the equilibrium concentration of the radical in a reversible system is not a fictitious, unreasonably small but rather a finite, well measurable quantity. A large equilibrium concentration of the radical has been clearly demonstrated for very acid solutions of cationic dyes, and for very alkaline solutions of anionic dyes. In the intermediate range the only evidence so far is the fact that the potentiometric titration curve is just a little bit steeper than that expected for a bivalent oxidation without intermediate step. Here the deviation, as expressed in terms of the index potential, usually exceeds the limits of error but little. In this paper we shall deal with cationic dyestuffs which are able to form semiquinones to an easily detectable amount not only in very acid, but also in alkaline, solutions. Hereby the interpolation of the behavior in the intermediate pH range around neutrality can be accomplished with fair accuracy. It will be shown that the index potentials obtained from this interpolation agree satisfactorily with those obtained directly by titration. Our experience with potentiometric titrations has led us to the conclusion that the index potentials experimentally derived are reliable to within ± 0.2 – 0.3 mv. The agreement between interpolation and experiment in the pH region around neutrality supports the conclusion that the values of 0.5 to 1.0 mv. above the theoretical minimum value of 14.3 mv. are certainly well beyond our experimental errors.

Those cationic dyestuffs for which free radicals can easily be detected not only in very acid but also in alkaline solution, are not new ones. They are oxazines and thiazines. The reason why no reliable titration curves in alkaline solutions could be obtained so far is the extremely low solubility of the uncharged free bases of some of the dyestuffs, such as thionine or oxonine, or the insolubility of the leuco dye together with the lability of the dye itself in alkaline solution, such as is the case for methylene blue. We shall begin with the

first of these two cases. Oxonine (3,9-diaminophenoxazin¹) was found to be the most suitable dyestuff to start the investigation, because its semiquinone formation constant in alkaline solution was found to be greater than for other comparable dyes. The artifice by which the difficulty arising from insolubility was overcome, consisted in using a buffer containing some organic solvent, such as alcohol or pyridine. Aqueous buffers containing 20% of pyridine were found very useful. To be sure, Geake and Lemon² have shown that the semiquinone formation constant may depend on the nature of the solvent. In the present case, however, and under our working conditions the influence of pyridine in the concentration used was found to be negligible. Comparative measurements of the normal potential and the index potential of oxonine, at pH around 6 in aqueous buffers and buffers containing 20% pyridine, or at pH around 11.5 with 10% or with 20% pyridine, showed no differences that might have been considered decidedly beyond the limits of error. By pH in a pyridine-containing buffer we understand the value calculated from the potential at the hydrogen electrode as though it were a purely aqueous solution. The following experiments accordingly fulfill a double purpose: first, to demonstrate the existence of semiquinones in alkaline solution, supplementing the former experiments in very acid solutions; and, second to utilize the data now available for acid and alkaline solutions to interpolate the behavior in neutral solution. One may thus compare the results obtained for approximately neutral solutions with those obtained previously by utilizing directly such index potentials as exceeded the minimum value very little, say, by 0.5 to 1 millivolt, so little indeed that their direct utilization for further calculations based upon them, may have been not quite convincing for some critics.

A. Oxonine

(a) *The Optical Properties of Oxonine and its Semiquinone.*—The free base of oxonine, as existing in solutions of pH > 11, is red and shows a very diffuse, broad absorption band around 500 mμ. The univalent cation which exists from pH 10 to 0, is blue-violet, exhibiting a gorgeous red fluorescence. It has a sharp and intense absorption band with peak at 575 mμ. In acetate buffer containing 20% pyridine, this band is displaced by about 15 mμ toward the red. The fluorescence band lies in the red and orange and has its center at about 600 to 610 mμ. The bivalent cation, as established in strongly acid solution has a sharp absorption band at 650. In almost concentrated sulfuric acid, a trivalent cation is formed, of dirty bordeaux red color, with a very diffuse and weak band around 550 and another region of diffuse absorption at the violet end of the visible spectrum.

(1) S. Granick, L. Michaelis and M. P. Schubert, (a) *THIS JOURNAL*, **62**, 1802 (1940); (b) **62**, 204 (1940).

(2) A. Geake and J. T. Lemon, *Shirley Inst. Mem.*, **16**, 11 (1938).

The univalent cation shows, with increasing concentration of the dye, a second band around 540, which, on comparison with analogous cases investigated by Rabinowitch and Epstein,³ may be interpreted as due to a dimerization ensuing from intermolecular interaction of the electric oscillators established by the quinone-benzene resonance. (On comparing the intensities of this second band for various concentrations (see Fig. 9 of the previous paper^{1a}) for oxonine with those of thionine, one sees that the polymerization is smaller for oxonine than for thionine (hence even very much smaller than for methylene blue). In the presence of pyridine, the polymerization is still smaller than in a purely aqueous solution. A quantitative estimate of the effect of this polymerization for the conditions of the following potentiometric titrations of

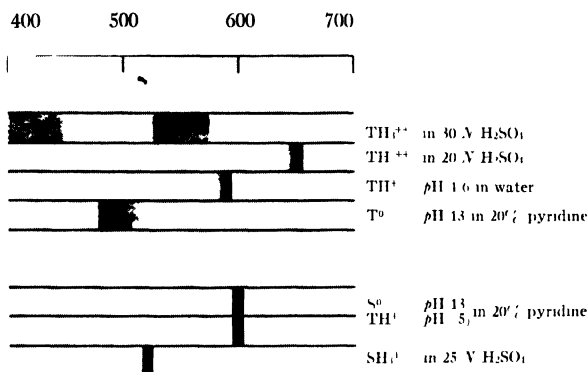


FIG. 1. The absorption bands of the various quinonoid (T) forms and semiquinonoid (S) forms of oxonine, schematically, as observed in the band spectroscopy.

oxonine has shown that it is entirely negligible for our working conditions, with respect to the evaluation of all constants within the scope of our concern.

When in a strongly alkaline solution (0.1 to 1 N sodium hydroxide) in 20% pyridine or alcohol, in a concentration of the dye sufficiently high to absorb almost all visible light, the color is diminished by gradually diluting with the solvent, the region of absorption shrinks more and more to the very diffuse band of the uncharged base, in the green and blue, and vanishes almost entirely in such concentrations as still appear pink for the unaided eye. When, however, the color of the concentrated solution is diminished, not by dilution, but by a reducing agent, a new and sharp band arises before fading is complete. Palladium-hydrogen, or sodium hydrosulfite or, more conveniently, because of the slowness of its action, glucose may be used as reducing agent. As the diffuse band of the free base recedes, a new sharp band around 590 mμ arises. It happens to coincide with the band of the univalent quinonoid cation

in 20% pyridine solution. Yet, this cation does not exist in such an alkaline solution, nor is there any fluorescence characteristic of it. The color, immediately prior to the complete fading of the red base, is blue-violet, without fluorescence. This band is that of the semiquinone. On oxidizing with air, this band vanishes entirely. The unusually high value of the semiquinone formation constant in alkaline solution for oxonine, together with its favorable optical properties, makes this dyestuff an especially favorable material for the desired studies.

It may be added as a preliminary note that the oxonine radical in alkaline solution also has been identified as a radical by its paramagnetism, as will be described in a further publication.

(b) *Structure of the Dye in its Various Levels of Oxidation and Ionization.*—There is no difficulty in writing down the four levels of ionization for the R form. With no proton attached, it is as in formula I. The first proton is attached at position 1, the second at position 2, the third at position 3. We may designate the four ionization levels of R as R^0 , RH^+ , RH_2^{++} , RH_3^{+++} . The first and second constant of ionization will differ from each other not very much more than postulated according to the statistical effect, the third must be very different. In fact, we have $pk_1 = 6.5$, $pk_2 = 5.5$, $pk_3 = -1.2$, approximately. The T-form, free of detachable protons, T^0 , can be written as in formula II. In acid solution TH^+ is formed. The proton is attached not to the $-\dot{N}H_2$ group, but to the $=\dot{N}H$ group (position 1) because of the resonance established hereby. This is the "quinone-benzene resonance," characteristic of almost all dyestuffs with a sharp band spectrum in the range of visible wave lengths.¹ In order to establish the lowest possible level of energy, the second proton in TH_2^{++} is attached to the bridge-N (position 2), whereby the resonance is maintained, as is the sharpness of the absorption band. In fact, the band of TH_2^{++} is of the same type as that of TH^+ , only displaced by 80 m μ toward the red. When TH_3^{+++} is formed by forcing upon the molecule a third proton, in strong sulfuric acid, at position 3, any quinone-benzene resonance is abolished, and the absorption spectrum is not only displaced toward the blue but is also very much more diffuse. The attachment of this third proton requires such an extremely acid solution because the NH_2 group to which it is attached is not a regular amino group, but one in resonance with a $=NH_2^+$ group.

The structures of the S-forms may be discussed as follows. Since there are three N-atoms capable of combining with a proton, we have to distinguish the ionization levels S^0 , SH^+ , SH_2^{++} and SH_3^{+++} . Judging from the parallelity of the E_1 , E_2 and E_m curves (Fig. 2) in strongly alkaline solution, the ionization

(4) As to the correlation of resonance and color in dyestuffs, see L. Pauling, *Proc. Natl. Acad. Sci.*, **25**, 577 (1939); G. Schwarzenbach, *et al*, *Helv. Chim. Acta*, **20**, 490 and 654 (1937); THIS JOURNAL, **60**, 1667 (1938).

level of S is easily obtained for $pH > 11$. It is S^0 . Two tautomeric forms of S^0 are imaginable, S^{0a} and b (III and V). In both of them, the electrons may be distributed in various ways; so several resonating structures can be written for both. Of these, two are especially worth mentioning, S^{0a} , α and β (IV) which represent an equivalent resonance system. Although the distribution of the electric charge here is less favorable than in S^{0a} (III) the resonance

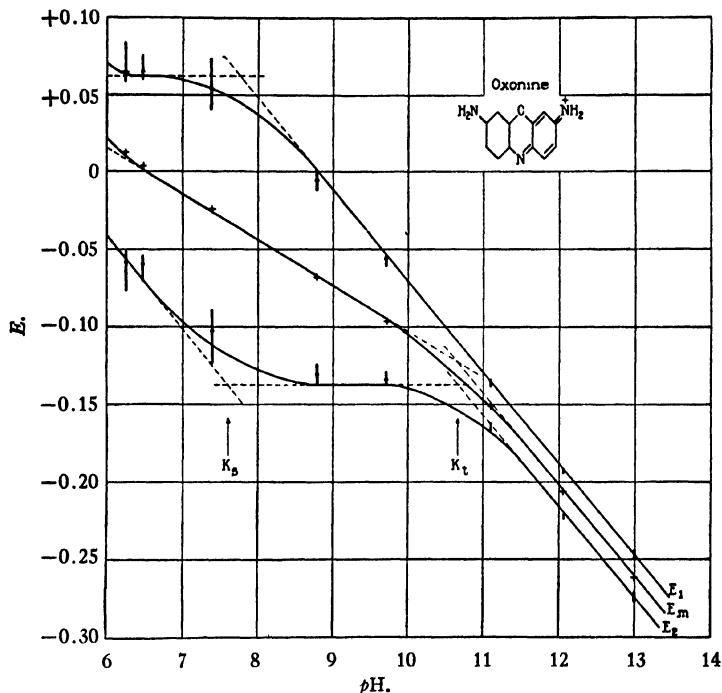
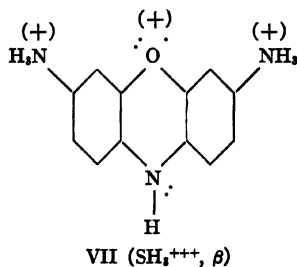
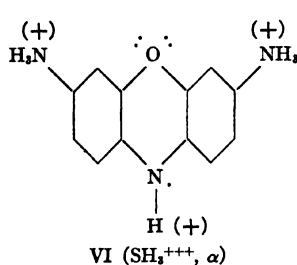
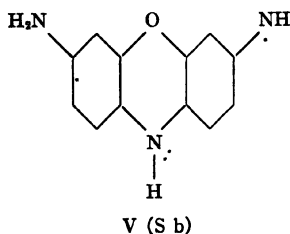
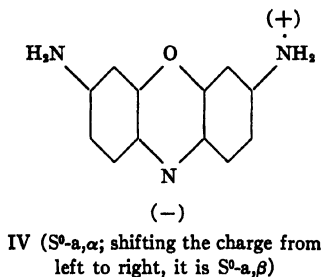
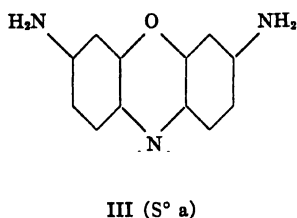
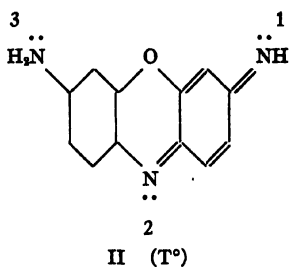
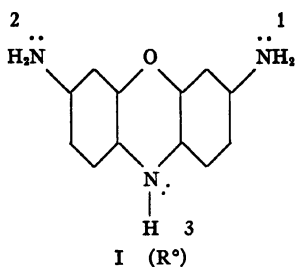


FIG. 2. The three normal potentials E_1 , E_m and E_2 , plotted against pH from 6 to 13, for oxonine. The lengths of the marks conform to a probable error in the index potential of ± 0.2 millivolt.

energy may be large enough to compensate for the separation of the charges. Since this type of resonance (IV) is the same as in TH^+ , one might expect the same type of absorption spectrum. In fact, the radical in the ionization level S^0 has the same absorption spectrum in the visible region as TH^+ , which exists in acid solution. A similar resonance for S^{0b} (V) is not imaginable. So it is suggestive to admit at least in part the resonance structure (IV) for the S^0 form.

The SH^+ form is imaginable in several tautomeric forms by attaching a proton to any one of the available N atoms in S^0 . As Fig. 3 shows, it can exist

only in a very low equilibrium concentration. Since there is no spectroscopic evidence for SH^+ either, it will not be worth while to discuss the various possible resonance structures of the various tautomers of SH^+ .



On proceeding to SH_2^{++} and SH_1^{++} , we encounter a new situation, which is similar for both. As can be seen from Fig. 3, SH_2^{++} is capable of existence in

equilibrium condition only to a minute extent. It is understandable therefore that spectroscopic evidence is missing. However, SH_3^{+++} can easily be detected spectroscopically. It is that form of the radical described in the previous paper (1, a and b) as existing in very acid solution. We restrict the discussion to this SH_3^{+++} form. It is formulated in (VI), in resonance with (VII). The profound change of type of resonance from that in the lower levels of ionization may seem a mere speculation at first glance. However, the following consideration lends support to such a suggestion, and even seems to make it quite unavoidable.

It has been shown that the spectrum of s-oxazine, the free radical derived from the unsubstituted skeleton of the oxazine dyes, such as arising from partial oxidation of oxazine in an acid solution, differs from the analogous s-thiazine (and also s-selenazine): s-oxazine has one sharp, very intense band, s-thiazine has a complicated series of less intense bands. Furthermore, as has been shown, all dyestuffs derived from oxazine by attachment either of one or of two amino groups, form in strongly acid solution a radical with the same absorption spectrum as does the unsubstituted s-oxazine, and all radicals of dyestuffs derived from thiazine, or selenazine, by attachment of one or two amino groups or even methylated or phenylated amino groups, have the very characteristic, unmistakable complicated spectrum of unsubstituted s-thiazine (or selenazine). It is evident that the side chains, if there be any, are not involved in the electric oscillator responsible for this spectrum. This is solely determined by the nature of the bridge atoms. So, the resonance of the SH_3^{+++} form of oxonine, may with good reason be assumed to be essentially of the same nature as that of the unsubstituted oxazine, and not influenced by the amino side-chains. In SH_3^{+++} both side chains are quaternary amino (not imino) groups and thus can supply no electrons for resonance across the molecule. This may be taken as sufficient evidence for the resonance structure $\text{VI} \rightleftharpoons \text{VII}$.

(c) *Potentiometric Titrations of Oxonine.*—The experiments are tabulated in Table I.

(d) *The Three Normal Potential Curves for Oxonine.*—Sufficient data are now available to construct a diagram of the three normal potentials for oxonine over a $p\text{H}$ range from almost concentrated sulfuric acid to $p\text{H}$ about 14 (Fig. 3). For the most acid range, the plot may be considered as a schematic one, the values of $p\text{H}$ and of the ionization constants being only approximately known. To evaluate $p\text{H}$ in this region, Hammett's acidity functions were taken⁵ as $p\text{H}$ values. The data obtained by Hammett were used by Hall and Spengeman⁶ for plotting the acidity function against molarity of sulfuric acid with the remarkable result that this plot beginning with 1 M acid, is a straight line over a wide range, with slope 0.5. This holds not only over the range

(5) Hammett and Paul, *THIS JOURNAL*, **58**, 827 and 830 (1934); **58**, 2182 (1936).

(6) N. F. Hall and W. F. Spengeman, *ibid.*, **62**, 2487 (1940).

as drawn by these authors, but up to 18 *M* sulfuric acid. This plot has been used for translating the various concentrations of sulfuric acid as used in terms of normality in the previous paper, into *pH* values.

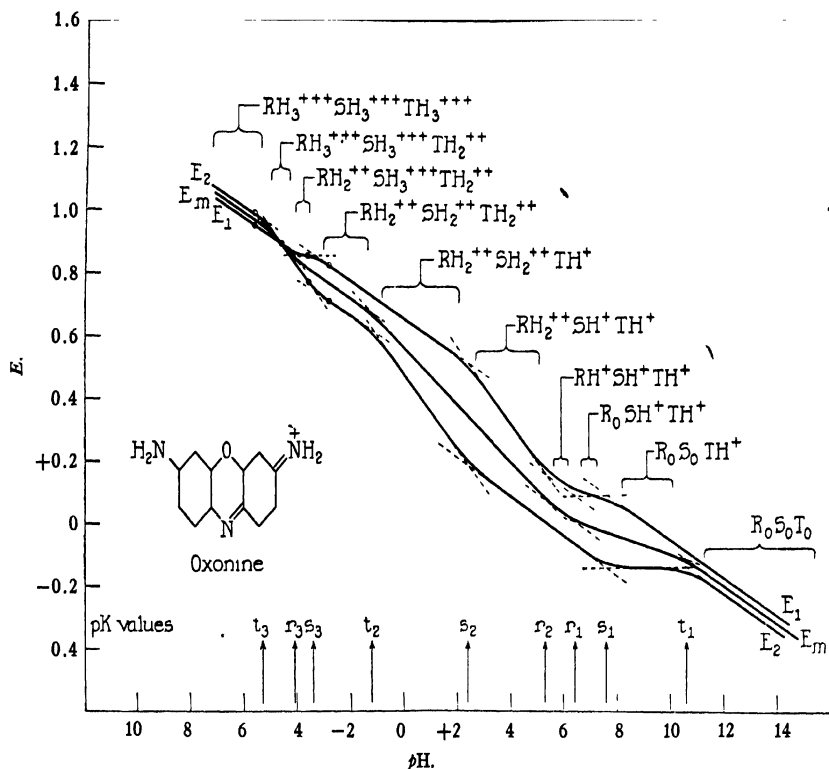


FIG. 3. The three normal potentials of oxonine plotted against the *pH* range from -8 to +14. The ionization constants of the R, S and T forms are marked by arrows. The experimental values used for plotting the region of high acidity are marked by circles.

The ionization constants K_{a2} and K_{a3} were determined optically as follows. A solution of the dye at *pH* 4.6, containing only TH^+ , and another solution in 15 *N* sulfuric acid, containing only TH_2^{++} were prepared. Then a solution of the dye in such a concentration of sulfuric acid was established, so that its color matched that of the optical mixture of TH^+ and TH_2^{++} in a color comparator, provided the total concentration of the dye was double that of either the pure TH^+ or the TH_2^{++} solution. The acidity function of this sulfuric acid concentration, was considered as its *pH*, and thus also as pK_{a2} . In an

analogous manner, pK_{13} was determined. No great accuracy is claimed for these values.

In this acid region the plot shows schematically in principle what one wants to know. On the other hand, the measurements presented in this paper yield rather accurate data for the plot in its alkaline region (Fig. 2). The alkaline end and the acid end of this plot can now be easily interpolated so as to fit to the number and most probable positions of the bends in the intermediate range. So one arrives at diagram Fig. 3. Those points in the E_1 and E_2 curve fixed by potentiometric and spectroscopic data, in the extreme acid region, are marked by circles.

Now we may compare the interpolated middle section with the results obtained directly from such index potentials which exceed the possible minimum value (14.3 mv.) by not more than 0.5 to 1 millivolt. It can be seen that there is a satisfactory agreement. We may add that in some experiments in the neighborhood of pH 0, the index potential was within the limits of error equal to its theoretical minimum value 14.3, which also agrees with Fig. 3. The bends corresponding to the ionization constants of the S-forms are herewith unambiguously fixed, if the whole picture is to become a coherent one. The accuracy of the ionization constants of S, of course, is not very great. However, the most interesting values, namely, those of the maximum ratio of semiquinone to total dye, $(s/a)_{max}$, are very little affected by such uncertainties in the ionization constants of the S forms. It may be added that the value of pK_{13} is rather arbitrary, but some incorrectness of its value has not much influence on the shape of the curve in the more accessible pH range.

It may seem striking that the attachment of the third proton in SH_3^{+++} , according to diagram Fig. 3, is easier than that of the third proton in TH_3^{+++} , the ionization exponents being -3 and -5.7 , respectively. The acceptance of this fact is unavoidable in order to bring about the convergence of the three potential curves ensuing in the crossing over at $pH -5.1$. Hereby the almost abrupt increase of the semiquinone formation constant in strongly acid solution is accounted for. The explanation of this fact may be this. The third proton of the T form is attached to a group which is partly a $-NH_2$ group, partly a $=NH_2^+$ group, because of the resonance. This makes this group a very weak basic one. In contrast, the third proton of the S form is added to a true $-NH_2$ group, which is, though weakly basic, yet not weaker than any third basic group of a trivalent base of similar structure.

B. Thionine and Methylene Blue.—Thionine is analogous to oxonine. The difference is essentially that the maximum value of the semiquinone formation constant, holding for strongly alkaline solution, is decidedly smaller than for oxonine. The highest index potential is 15.6 mv. (instead of 19 for oxonine). The excess above the theoretical minimum value, 14.3, is distinctly beyond the limits of error. The difference from the behavior of oxonine might tentatively

TABLE I
Oxonine, 1×10^{-4} Molar

| Buffer, 100 cc. of buffer consisting of 20 cc. pyridine and the aqueous buffers: | pH of the final buffer | E_m potential in volts, at 50% oxidation | E_i index potential in millivolts | | Method |
|----------------------------------------------------------------------------------------------|------------------------|--------------------------------------------|-------------------------------------|------------------|--------------------------------------------------------------|
| | | | 25-50% oxidation | 50-75% oxidation | |
| (1) Acetate buffer, ionic strength 0.1 (pH without pyridine 4.45) | 6.26 | +0.0125 | 14.8 | 14.8 | Red. with Pd + H_2 , titr. with $K_3Fe(CN)_6$ |
| (2) Acetate buffer, ionic strength 0.1 (pH without pyridine 4.62) | 6.49 | + .0035 | 15.0 | 15.0 | Same |
| (3) 45 cc. $M/15$ KH_2PO_4 + 25 cc. $M/15$ Na_2HPO_4 ; final volume filled up with water | 7.42 | -.0241 | 14.9 Av. 14.75 | 14.6 | Same |
| (4) 2.07 g. sodium veronal; 2.5 cc. 1 N HCl, filled up with water | 8.79 | -.0685 | 15.0 | 15.0 | Same |
| (5) 1.50 g. dimethyl glycine + 5.4 cc. N HCl; filled up with water | 9.72 | -.0965 | 16.1 | 16.1 | Red. titrn. ^a with leucorosindulin GG |
| (6) 60 cc. $M/15$ Na_2HPO_4 + 10 cc. $N/10$ NaOH; the rest water | 11.11 | -.1511 | 18.9 | 18.6 | Red. with Pd + H_2 , ^b titr. with $K_3Fe(CN)_6$ |
| (7) 45 cc. $M/15$ Na_2HPO_4 + 30 cc. $N/10$ NaOH, the rest water | 12.05 | -.2065 | 19.0 | 19.0 | Same ^b |
| (8) 10 cc. 1 N NaOH; the rest water | 13.05 | -.2617 | 17.5 | 19.2 | Same ^b |
| | | | Average 18.35** | | |

^a This dyestuff, almost indispensable for reductive titrations because of its very negative potential range and its stability over a wide pH range, is no longer commercially available. It can be prepared from Rosinduline 2B (National Aniline and Dye Company) as follows. A solution of 5 g. of this dye in 60 cc. of water is heated in a sealed tube at 180° for twenty-four hours. Orange rosetts of the free Rosindon-monosulfonic acid appear on cooling. Acidify the solution with 10 cc. of 2 N HCl. Filter off the precipitate. If the starting material is good, the filtrate is practically colorless; yield 1.9 g. or 70%. Convert this acid into its well-crystallized sodium salt (Rosindulin GG) by suspending in 200 cc. of water and adding sodium carbonate just to neutralization. Add 2 g. of sodium chloride, heat to boiling, cool twenty-four hours at room temperature, filter, wash with very little 1% sodium chloride solution. Calculated for the dry sodium salt: N, 6.60; S, 7.55. Found N, 6.65; S, 7.6. Potentiometric titration of its leuco dye with $K_3Fe(CN)_6$ in acetate buffer pH 4.62 shows normal potential -0.1298 volt, with index potential 15.1; 15.2 millivolts.

^b In these alkaline solutions the potential at the end-point drifts back due to a further slow irreversible oxidation. This causes a slight indefiniteness of the end-point only at pH 13, hence the slight apparent asymmetry of the index potential in experiment No. 8.

TABLE II

Correlation of pH ; E_s (Index Potential); $E_2 - E_1$ (Difference of the Normal Potential of the More Positive and the More Negative Univalent Partial Oxidation-Reduction System); $\log k$ (k = Semiquinone Formation Constant) and $(s/a)_{\max}$ (Maximum Ratio of Semiquinone to Total Dye)

Approximately interpolated values according to Fig. 3

| pH | 12 | 10 | 8 | 6 | 4 | 2 | 0 | -2 | -4 | -6 |
|---------------------------|-------|-------|------|------|------|------|------|------|-------|-------|
| $E_2 - E_1$ | -24 | -46 | -182 | -110 | -230 | -315 | -180 | -120 | -40 | +35 |
| E_s | 19 | 17 | 14.6 | 14.9 | 14.4 | 14.3 | 14.6 | 15.1 | 17.7 | 28 |
| $\log k$ | -0.40 | -0.76 | -3 | -1.8 | -3.8 | -5.2 | -3 | -2 | -0.67 | +0.51 |
| $(s/a)_{\max} \times 100$ | 23 | 17 | 1.6 | 5.7 | 0.6 | 0.12 | 1.5 | 4.7 | 19 | 50 |

TABLE III

Thionine, 1×10^{-4} Molar

| Na_2HPO_4 , $M/15$, cc. | Buffer | | | pH | E_m | E_s | | Method |
|---------------------------------------------|----------|--------------|--------------|-------|---------|-------------------|-------------------|--------------------------------------------------------------------------------|
| | NaOH, cc | Pyridine, cc | Water to cc. | | | 25%-50% Oxidation | 50%-75% Oxidation | |
| 60 | 7 0.1 N | 20 | 100 | 10.84 | -0.0687 | 15.6 | 15.6 | Reduced with PdH_2 , titrated with $\text{K}_3\text{Fe}(\text{CN})_6$ |
| 60 | 10 0.1 N | 20 | 100 | 11.16 | -0.0867 | 16.0 | 16.0 | |
| 60 | 13 0.1 N | 20 | 100 | 11.34 | -0.0931 | 15.7 | 15.5 | |
| — | 10 1 N | 20 | 100 | 12.93 | -0.1852 | 16.1 | 16.3 | |

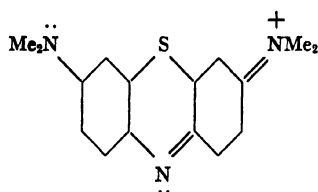
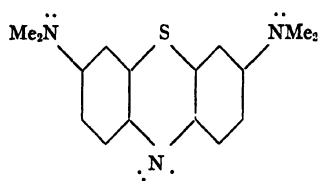
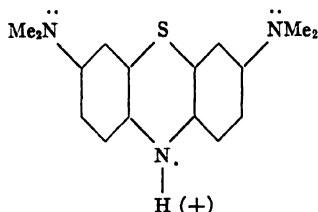
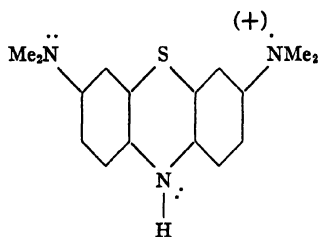
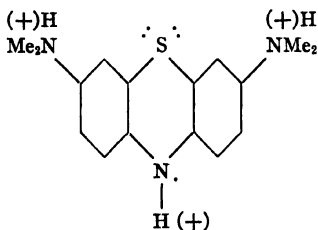
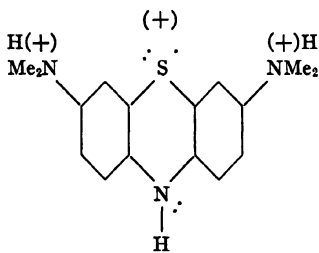
TABLE IV

Methylene Blue, 1×10^{-4} Molar

| Buffer | pH | E_m | E_s | | Method |
|----------------------------------------------------------------------------------------------------------------------------------|------|-----------|-------------------|-------------------|----------------------------------------------------------------------------------------|
| | | | 25%-50% Oxidation | 50%-75% Oxidation | |
| 1 35 cc. $M/15$ KH_2PO_4 35 cc. $M/15$ Na_2HPO_4 20 cc. pyridine Water to 100 cc. | 7.56 | +0.0020 | 14.8 | 15.0 | Reduced with $\text{Pd} + \text{H}_2$ titrated with $\text{K}_3\text{Fe}(\text{CN})_6$ |
| 2 25 cc. $M/15$ KH_2PO_4 25 cc. $M/15$ Na_2HPO_4 50 cc. absol. ethanol Water to 100 cc. | 7.70 | +0.0110 | 14.8 | 14.7 | Reduced with $\text{Pd} + \text{H}_2$ titrated with $\text{K}_3\text{Fe}(\text{CN})_6$ |
| 3 Same as no. 1. | | +0.0022 | 15.1 | 15.2 | Red. titrn. with Leuco-Rosindulin GG |
| 4 1.017 N H_2SO_4 | 0.36 | (+0.5025) | 14.0 | 13.9 | Red. with $\text{Pd} + \text{H}_2$, titr. with $\text{K}_2\text{Cr}_2\text{O}_7$ |

be accounted for by assuming pK_s to be about 8.0 (instead of 7.5). Such a slight difference would be sufficient to turn the whole situation, so easy to

decipher for oxonine, into one much more difficult to recognize. No convincing spectroscopic evidence could be obtained for the thionine radical in alkaline solution. In part it may be due to the fact that the maximum ratio of semi-quinone is only 8% for thionine, whereas it is 23% for oxonine. It is possible,

VIII (T⁺)IX (S⁰) (there is no evidence for its existence)X (SH⁺, α)XI (SH⁺, β and (reversing right and left) γ)XII (SH₃⁺⁺⁺ α)XIII (SH₃⁺⁺⁺ β)

in addition, that the band of the radical overlaps more with that of the uncharged base of the quinonoid dye.

In methylene blue, the situation is somewhat different. For a quaternary base such as methylene blue, no ionization level corresponding to S⁰ exists. The various ionization levels of the T forms may be distinguished as T⁺ (formula VIII), TH⁺⁺, TH₂⁺⁺⁺. No form corresponding to S⁰ (IX) can be observed. The forms occurring are SH⁺ (X and XI), SH₂⁺⁺ (no formula drawn), and SH₃⁺⁺⁺ (XII and XIII). The essential difference in the plots

of oxonine and methylene blue (Fig. 4) ensues from the non-existence, at least within the reachable pH range, of the form S^0 . This may be accounted for by the fact that methylene blue in alkaline solution is so unstable that no reliable index potentials can be obtained. The half-schematic plot (Fig. 4) has been, for this reason, extended only over the pH range up to 7. The interpolation method, so instructive in the case of oxonine, cannot be applied for methylene blue, and we have to rely on the index potentials even in the pH region around neutrality. The large concentration effect for methylene blue indicating dimerization of the T-form makes the accurate determination of the limiting value of the index potential for infinitely low concentration more difficult than for the other dyes. According to the many experiments per-

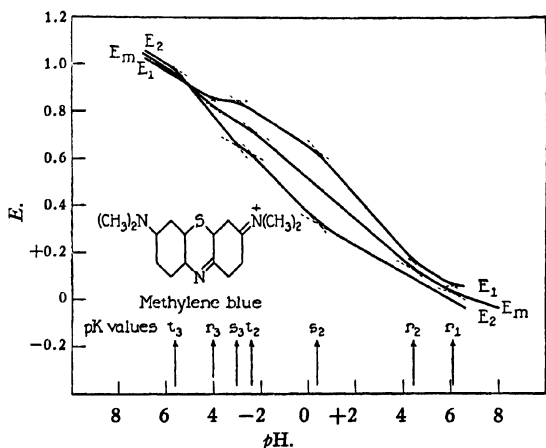


FIG. 4. The three normal potentials of methylene blue plotted against pH , holding for very dilute solutions of the dyestuff.

formed during the past years the value $E_i = 14.9 \pm 0.2$ for pH 4.6 does not seem too high. Titration experiments in solutions of higher concentration than $1 \times 10^{-4} M$ distinctly show the effect of polymerization. In the first place, the normal potentials are displaced toward the negative side as shown already by Clark, Gibbs and Cohen.⁷ Secondly, the index potentials are changed with increasing concentration. This change is two-fold: they become smaller (values down to 9 mv. could be reached), and the two index potentials (that from 50 to 25%, and that from 50 to 75% titration) become unequal. Without going into the details for the time being, the authors are convinced that 14.9 ± 0.2 is not too high as a limiting value for the index potential. Utilizing this value and those obtained for very acid solutions in the previous paper,^{1b} one arrives at the diagram Fig. 4, which does not claim to be very

(7) W. M. Clark, H. D. Gibbs and B. Cohen, *U. S. Publ. Health Repts.*, **40**, 1131 (1925).

precise, yet at any rate may contribute a good deal to the notion of this frequently yet never exhaustively studied dyestuff.

SUMMARY

Oxazine forms visually detectable semiquinone radicals not only in very acid solutions, as has been shown recently, but also in alkaline solution. Oxonine is the most suitable dyestuff for accurate measurements of the semiquinone formation constant over the whole pH range from -8 to $+14$. From the reliable measurements in alkaline solution, and the relatively fairly accurate measurements in strongly acid solutions, the values for the intermediate pH range can be interpolated. The values thus obtained for the intermediate pH range agree within the limits of error with those directly obtainable from the slopes of the titration curves. We need not now consider the latter as less reliable within this intermediate pH range. The plot Fig. 3 for oxonine may be said to be the most complete set of data for any dyestuff yet investigated, since it includes the three normal potentials, the ionization constants of all the three levels of oxidation, and the semiquinone formation constants over the whole pH range. The analogous behavior of thiazine dyestuffs is also discussed.

AUTOMATIC SPEED CONTROL FOR THE AIR-DRIVEN ULTRACENTRIFUGE

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(Received for publication, March 21, 1941)

A speed control for the air-driven ultracentrifuge based on a stroboscopic principle is described. The pressure of the air driving the centrifuge is automatically decreased or increased according as the centrifuge tends to accelerate or decelerate. The speed of the centrifuge can be maintained constant within less than one revolution a second.

The accuracy with which the molecular weights of large molecules, such as proteins, can be obtained with the ultracentrifuge depends, among other factors, upon a precise knowledge of the speed of rotation, and upon maintaining that speed constant during the time necessary to make a determination. It might appear that the speed of an air-driven ultracentrifuge should not vary if the pressure of the air driving the turbine remains constant and that a device for maintaining a constant air pressure should prevent variation in the speed of the turbine. The speed is, however, influenced by such factors as small variations in the pressure of the residual air in the evacuated chamber as well as by temperature fluctuations affecting the viscosity of the lubricating oil. It is desirable, therefore, that the operation of the valve which controls the air pressure should be determined by the speed of the centrifuge. With this in mind, an automatic optical speed control for the air-driven ultracentrifuge based on a stroboscopic principle was constructed and is described in this article. The device is simpler than the control recently described by Björnståhl¹ and proceeds from an entirely different principle.

In the new control device the knob K of Figs. 1 (a) and 1 (b) projects from the upper surface T of the air turbine that drives the rotor. One face of the protruding central knob K is polished and acts as a mirror M , the rest of the knob being blackened. A discontinuous light source of known and variable flashing speed is represented at L . A Strobolux unit of the General Radio Company has proved quite satisfactory. The light source is focused by a condensing lens C on the photoelectric cell E_1 or E_2 (RCA tubes, 922) depending on the position of the mirror M . If the rotation of the turbine is clockwise and the number of revolutions per second is identical with or a multiple of the frequency of the flashes, then the turbine appears motionless. On the other

¹ Y. Björnståhl, Rev. Sci. Inst. 10, 258 (1939).

hand, the turbine appears to rotate slowly clockwise if the frequency or a multiple of the frequency of the turbine is slightly higher than that of the flashes and vice versa. The action of the control is as follows: The light strikes the photoelectric cell E_1 or E_2 each time the mirror is at the proper angle. By appropriate circuits the photoelectric current generated in E_1 starts the motor M_1 (Figs. 1 and 2) which in turn opens the valve V of the air line driving the centrifuge. The photoelectric current generated in E_2 starts the motor M_2 which closes the same valve. If the turbine rotates slightly faster than the desired speed, as determined by the frequency of the light source, then the beam of light which appears to rotate clockwise first strikes the photoelectric

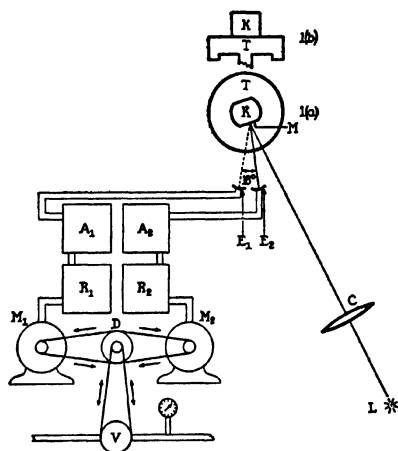


FIG. 1. Schematic representation of the action of the speed control. $E_1 = E_2$ = photoelectric cell; $A_1 = A_2$ = amplifier; $R_1 = R_2$ = relay system; $M_1 = M_2$ = motor; D = differential transmission; V = air valve.

cell E_2 (Fig. 1) and operates motor M_2 . However, the same beam of light strikes photoelectric cell E_1 a moment later. It is therefore necessary to provide a relay system which prevents the operation of the corresponding motor M_1 and limits the period of operation of the motor M_2 . The details of this relay system are shown in Fig. 2. The effect of the photoelectric current generated in E_2 is to decrease the plate current of the last tube of a two-tube amplifier at the right of the figure, thereby opening relay R_2 . (With d.c. 220 volts available, a d.c. amplifier with a 36 tube in the first stage and a 6J5G tube in the second proved satisfactory.) The opening of R_2 increases the voltage of the grid of the thyatron T_2 which in turn permits the current to flow through the plate circuit. The coils of the relay r_1 and the motor M_2 are connected in series in the plate circuit of T_2 . Under the influence of the plate

current of T_2 , the motor M_2 starts, slowly reducing the air pressure and thus the speed of the centrifuge. Simultaneously the relay r_1 closes the circuit of the control grid of T_1 , thus preventing motor M_1 from operating. If we assume that the turbine rotates slightly slower than the desired speed, then the photoelectric cell E_1 is first struck by the light and motor M_1 instead of motor M_2 starts.

Since the grid of the thyatron does not control the plate current once started, the motors M_2 or M_1 would run indefinitely if it were not for the following device which interrupts the plate current of either T_1 or T_2 every 15 seconds by

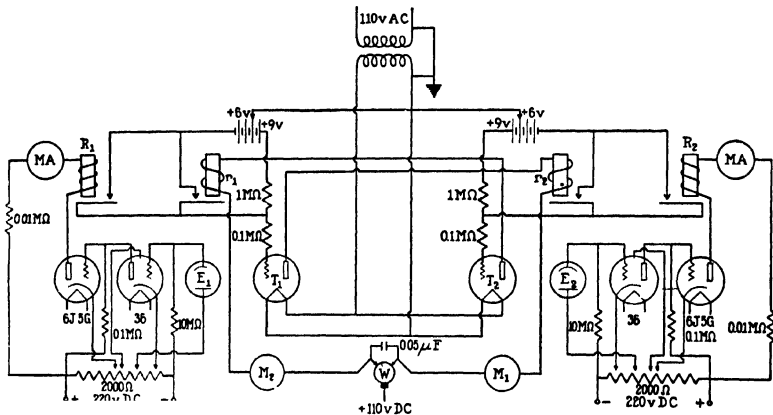


FIG. 2. Wiring diagram of the photoelectric cell circuits, amplifiers and relay systems. $r_1 = r_2$ = Ward Leonard relay 0.35A, 17 Ω ; $R_1 = R_2$ = Western Electric relay J-20 (a) 0.004A, 1600 Ω ; $T_1 = T_2$ = thyatron tube FG 57; $E_1 = E_2$ = RCA 922.

shutting off the voltage supply of the plate for about 0.1 second, thus permitting the grid to regain control of the tube.

The motors M_1 and M_2 , geared down to a ratio of 1/595, rotate the shaft A of Fig. 3 through the differential transmission at a speed of 4 r.p.m. The shaft holds at one end a metal wheel W , also shown diagrammatically in Fig. 2. The current instead of going directly to the motors first flows through a commutator on the wheel, contact being made by two brushes held against the edge, and the circuit is broken for about 0.1 second during each revolution of the wheel. Hence, every 15 seconds the motor which is operating stops, making the system once more subject to control, since the current through the operating thyatron has been interrupted. The air pressure will thus be increased or decreased depending whether photoelectric cell E_1 or E_2 is hit first by the beam from the stroboscope. During the 15-second intervals, when one of the two motors is operating, the passage of the beam of light in front of the photoelectric

cells has no influence on the mechanism. However, every 15 seconds the instrument is able to differentiate between a levo-rotation and a dextro-rotation of the beam of light. The instrument operates in the wrong direction when this beam of light happens to strike within the angle E_1-K-E_2 , of Fig. 1, at the time the regulating system regains control after stopping of motor M_1 or M_2 . Since this angle is about 18° the probability of such an event is, however, only $18/360$ and practically does not interfere with a satisfactory control of the speed since the "mistake" is immediately corrected.

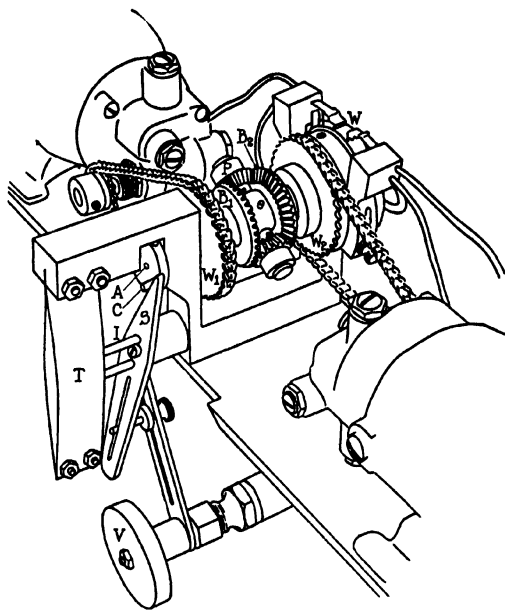


FIG. 3. Mechanism by which the air valve is opened or closed.

The motors operate the air valve through a differential gear which can be seen in Fig. 3. The cogwheel W_1 is fastened to the bevel gear B_1 and W_2 is fastened to B_2 . The cogwheels and bevel gears are free to rotate on the shaft. The direction of rotation of the motors being opposite, this arrangement allows their motion to be transmitted to the same shaft A which will turn clockwise or counterclockwise depending on which motor is operating. If, for instance, the opening motor M_1 is operating, then the bevel gear B_1 rotates counterclockwise and its motion is transmitted to the shaft through the pinion P fastened to the shaft. The end of the shaft carries a cam C which in turn makes the arm S rotate. The rotation of this arm S opens the valve V . If motor M_2 is

operating the motion transmitted to the valve *V* through the arm *S* is of opposite direction and the valve closes. Two steel blades *T*, acting as a spring, are held in front of the arm *S*. They are separated by two pins *I* fastened to *S*. When the arm rotates, the distance between the blades *T* is increased on account of the two pins *I*. As soon as the cam is released, the arm and hence the valve regain their original position due to the spring action of the blades. In other words, the valve is opened or closed for the period (about 5 seconds) during which the cam is engaged. At the beginning of an experiment, the valve is adjusted by hand to the required average pressure needed and the control will increase or decrease the pressure every 15 seconds from this average value according as the tendency of the centrifuge is to accelerate or decelerate.

It has been found that a variation of one pound at a time, in the operating pressure of 20 to 25 pounds, is necessary for a satisfactory control between 20,000 to 40,000 r.p.m. At 60,000 r.p.m. a variation of 1.5 pounds is more effective.

With this control, the speed of the centrifuge can be maintained equal to the flashing speed or a multiple of it within less than one revolution per second. The centrifuge runs are generally made at speeds which are multiples of 3600 r.p.m. in order to operate the Strobolux directly on the a.c. line frequency. Under these conditions, the speed of the centrifuge is independent of the calibration of the Strobolux and is kept constant for hours within less than one revolution per second.

This apparatus has been in operation for several months and has proved entirely reliable.

I wish to thank Dr. D. A. MacInnes for helpful suggestions for the presentation of this article. I am indebted to the Institute instrument maker, Mr. J. Blum, for the suggestion of the use of the differential transmission, and for the construction of the apparatus, and also for the assistance of Mr. John Ogilvie.

THE INFLUENCE OF GENETIC CONSTITUTION UPON THE INDUCTION OF RESISTANCE TO TRANSPLANTABLE MOUSE TUMORS

By MORRIS K. BARRETT*

(From the Laboratories of The Rockefeller Institute for Medical Research)

The induction of resistance to transplantable tumors in mice by a prior inoculation of homologous normal living cells was an early contribution to the study of cancer. Bashford, Murray, and Cramer (1, 2, 3, 4) reported the use of defibrinated blood to induce resistance by this method. Although it has long been recognized that the injection of homologous living cells has no effect on the development of spontaneous (5, p. 84) or induced (6, p. 18) tumors, nor will it influence the course of an established transplantable tumor (1), repeated attempts have been made to relate this phenomenon to control of the natural disease.

The mechanism involved has been the subject of extensive investigations, but no generally accepted explanation has been evolved. Murphy (7) has suggested that the phenomenon may be due to a type of sensitization and has adduced some evidence in support of such a conception. According to this view, the initial injection of normal cells sensitizes the animal to the subsequent inoculation of tumor cells in such a way that the resisting mechanisms of the host, whatever they may be, are caused to act with enhanced force. Whether this represents a cellular immunity in the ordinary sense remains an open question, but it should be kept in mind that the "sensitization" here is directed against certain attributes of intact cells which depend upon their viability and integrity, and is not just a matter of specific proteins or other cell constituents. This interpretation presupposes a degree of genetic difference between the cells injected and the host as a basis for the reaction. Such opinion is supported by the failure to stimulate resistance by injection of autologous cells (8; 9, p. 150) or to influence the take of an autograft by prior injection of homologous normal cells (10; 11, p. 540). Furthermore, the injection of defibrinated blood of an alien species will not influence the transplantation of mouse tumors into mice (12).

Now that strains of mice are available which are relatively, if not entirely, homogeneous, it is possible to test the effect of genetic differences upon this

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phenomenon and to make some inferences with regard to the influence of genetic homogeneity. It is important to do so because there is little chance of applying the principles involved to the control of the naturally occurring disease, if the mechanism proves to be a sensitization phenomenon.

If leukemia represents essentially the same pathologic process as cancer, a view now taken by many, there already exist some studies along these lines. Rhoads and Miller (13) showed that resistance to transplantable leukemia may be induced in mice by the injection of intact homologous cells; and MacDowell, Potter, and Taylor (14) presented evidence that, when embryonal tissues were used as the immunizing agent, the degree of resistance induced is dependent upon the genetic relationship of the donor and the recipient. These latter workers adduced further that the effect attained was not directly related to the susceptibility of the strain from which the donor came.

The work presented here was undertaken in order to observe some effects of genetic differences upon the phenomenon under discussion. This was done by comparing the growth of grafts of three transplantable mammary carcinomas in mice of three strains. Test mice of each strain were immunized with blood of their own strain, or with that of the other strains, and the results compared with each other and with those obtained with control animals. The material selected is not ideal for complete elucidation of the problem, but the results are significant and dependable within their range. The technique used was simple and is described in the following section.

Materials and Methods

Young animals of the Rockefeller Institute strain, of Strong's A strain, and of the C57 Black strain were selected as hosts. The Rockefeller Institute strain has been derived by mass inbreeding of an original stock comprising approximately 600 animals equally distributed among 3 albino strains—the Lathrop, the Bagg, and the Uppercu. All were obtained in 1917, and no new animals have been added since. The Rockefeller Institute strain was taken as a genetically indifferent strain which presented, nevertheless, much uniformity. The strain A mice were obtained in weekly consignments from the Roscoe B. Jackson Memorial Laboratory. The strain is a highly inbred albino one that has undergone brother-by-sister inbreeding since 1918. The C57 Black mice were also obtained from the Roscoe B. Jackson Memorial Laboratory. This is a strain which has been inbred by brother-sister matings for more than 20 generations. However, it is possible that animals from more than 1 subline were included in those used.

All hosts were from 4 to 6 weeks old, and their average weight was 12 to 16 gm. Most of the animals were males, but the sexes were not segregated.

Three transplantable mammary carcinomas were employed. These were (1) the well-known Bashford No. 63, here taken as a genetically indifferent

tumor; (2) the tumor known as 15091-A, a low-factor tumor which arose in an A strain female at Bar Harbor and is transplantable outside the parent strain (Cloudman (15)); and (3) tumor 755 which originated in a C57 Black mouse in the colony of Dr. H. J. Bagg, to whom the writer is indebted for this material. The tumor last mentioned was ill-suited to this work but was used in the absence of better material. It could not be used outside the strain of origin but did give some information within that strain.

Blood was obtained, with aseptic precautions, from the heart of old adults of each strain and defibrinated by shaking with glass beads. The nuchal region of the host was shaved, and 0.2 cc. of the defibrinated blood was injected subcutaneously.

The mice of each strain were divided into groups by random selection. The first group of each was given blood taken from A mice, the second group received blood from Rockefeller Institute mice, the third group received blood from C57 Black mice, and the fourth or control group received no blood. Some groupings were omitted in individual experiments for lack of material. Precautions against subconscious bias in observing the results were taken by indexing the random distribution on a separate record which was not again consulted until the completion of the experiment.

After the passage of 10 days all mice received a graft (approximately $3 \times 3 \times 2$ mm.) of healthy carcinoma tissue inoculated by means of a trocar into the groin. The growth of the grafts was recorded weekly in terms of caliper measurements. Final results were evaluated after 2 weeks' growth. The tumors were observed for 2 weeks beyond the initial period in all cases, but the figures given below reflect the number of tumors present at 2 weeks. Little or no difference would be seen if calculations were based upon a longer period of observation; but since the investigation here is limited to the initial establishment of a graft, it was thought that the earlier data served the purpose best. This is in keeping with a belief that, though there may be common factors, the primary take of a transplantable tumor is not necessarily dependent upon the same factors as is the subsequent growth of the tumor.

The work includes 27 experiments, using a total of 768 animals as hosts.

RESULTS

The results obtained revealed wide fluctuations in susceptibility of the animals. The variations are such that, even in this somewhat limited material, almost the entire range of susceptibility is covered.

Table 1 summarizes the data on all mice that were grafted with tumor 15091-A, the A strain tumor. In Rockefeller Institute mice grafted with this tumor the variations were very marked. It will be noticed that the greatest resistance was found in those animals which had been immunized with blood homologous to the tumor. For convenience the word "homologous" is used

throughout the remainder of this paper in the special sense of "having the same or similar genetic constitution."

Under the heading "Strain A" in table 1 are shown the results of inoculation of immunized animals of an inbred strain, A, with a tumor (No. 15091-A) which originated in that strain. The variation shown is not significant statistically. It may be observed that when genetically homogeneous mice are used, resistance to a homologous tumor cannot be induced by this method.

TABLE 1
Results of Inoculating Immunized and Control Mice with Tumor No 15091-A
(An A Strain Tumor)

Rockefeller Institute Mice

| Strain used as source of blood for immunization | Total mice | Susceptible | | Resistant | |
|-------------------------------------------------|------------|-------------|------|-----------|------|
| | No | No | Pct. | No | Pct. |
| A | 30 | 2 | 7 | 28 | 93 |
| Rockefeller Institute | 30 | 5 | 17 | 25 | 83 |
| C57 Black | 29 | 22 | 76 | 7 | 24 |
| None (Controls) | 29 | 29 | 100 | 0 | 0 |

Strain A Mice

| | | | | | |
|-----------------------|----|----|-----|---|---|
| A | 30 | 28 | 93 | 2 | 7 |
| Rockefeller Institute | 29 | 28 | 97 | 1 | 3 |
| C57 Black | 30 | 30 | 100 | 0 | 0 |
| None (Controls) | 30 | 30 | 100 | 0 | 0 |

C57 Black Mice

| | | | | | |
|-----------------------|----|----|----|----|----|
| A | 28 | 4 | 14 | 24 | 86 |
| Rockefeller Institute | 30 | 2 | 7 | 28 | 93 |
| C57 Black | 29 | 25 | 86 | 4 | 14 |
| None (Controls) | 28 | 27 | 96 | 1 | 4 |

The results of inoculating C57 Black mice with tumor 15091-A are also given in table 1. This represents the case of a pure-line host with a tumor which originated in a different pure line. The resistance induced by blood which is homologous to the tumor, i. e., strain A blood, is again great though not quite so great as that induced by Rockefeller Institute blood. However, the difference between these two is not significant statistically ($P = 0.34$), and the important observation is the effect of the A blood. It is interesting to note here that, according to Strong, one of the ancestral parents of the A strain was a Bagg albino, a strain which also enters into the Rockefeller Institute strain. It is also noted that in these relatively pure line hosts the homologous blood had little or no effect in inducing resistance against a heterologous tumor.

Table 2 presents the results of transplanting a genetically indifferent tumor

(Bashford No. 63) into an indifferent strain (Rockefeller Institute) and into a homogeneous strain (A).

It will be seen that in strain A mice the percentage of takes in the group given blood from the same strain was reduced to 77 percent of that in the controls while the incidence in the group immunized with blood from the other strain was reduced to 50 percent of that in the controls. In the Rockefeller mice the incidence of takes in the group immunized with blood from the same

TABLE 2

Results of Inoculating Immunized and Control Mice with Bashford No. 63 (A Genetically Indifferent Tumor)

Rockefeller Institute Mice

| Strain used as source of blood for immunization | Total mice | Susceptible | | Resistant | |
|-------------------------------------------------|------------|-------------|------|-----------|------|
| | No. | No. | Pct. | No. | Pct. |
| A | 44 | 2 | 5 | 42 | 95 |
| Rockefeller Institute | 48 | 14 | 29 | 34 | 71 |
| None (Controls) | 49 | 24 | 49 | 25 | 51 |

| Strain A Mice | | | | | |
|-----------------------|----|----|----|----|----|
| A | 83 | 50 | 60 | 33 | 40 |
| Rockefeller Institute | 88 | 34 | 39 | 54 | 61 |
| None (Controls) | 74 | 58 | 78 | 16 | 22 |

TABLE 3

Results of Inoculating Immunized and Control C57 Black Mice with Tumor No. 755 (A C57 Black Tumor)

| Strain used as source of blood for immunization | Total mice | Susceptible | | Resistant | |
|-------------------------------------------------|------------|-------------|------|-----------|------|
| | No. | No. | Pct. | No. | Pct. |
| A | 10 | 9 | 90 | 1 | 10 |
| C57 Black | 10 | 10 | 100 | 0 | 0 |
| None (Controls) | 10 | 10 | 100 | 0 | 0 |

strain was reduced to 59 percent of that in the controls, whereas a comparable figure for the group given blood from the other strain was 10 percent. It should be noted that the effect of homologous blood, as measured by relative reduction in percentage of takes, was less in the case of the A strain.

The results of inoculating C57 Black mice with tumor No. 755 are given in table 3. Here again is evidence that no effective resistance can be induced in a pure-line mouse against a tumor that is genetically homologous to the host.

Careful analysis of the data failed to disclose anything in the size of the tumors which could be correlated with the treatment of the animals, nor was

anything of interest discovered by noting the rate or the time of death among the animals in groups having a high incidence of takes.

Statistical analysis of the data indicates that the differences observed were definitely significant.

DISCUSSION

It must be emphasized that the discussion here is limited to influence upon the establishment of a graft. It is believed that after this initial period, when the tumor has become established, a new set of conditions prevail and that the factors governing the two periods may differ in part, if not altogether. This is not to say that there is no relation between the phenomenon under discussion and certain aspects of the natural disease, e. g. extension and metastasis.

All the data given in the tables are summarized in figure 1. All results in a given strain of mice are arranged in a horizontal row, while similar categories within strains are arranged in vertical columns. Some interesting differences can be seen at a glance.

It is apparent that, with the methods employed here, the genetic interrelationships of the materials used have an important influence upon the degree of resistance induced. The variations are so great that, even in this limited material, one may select conditions which will give almost any degree of resistance, ranging from nearly complete immunity to complete susceptibility. The inferences from this with regard to other types of experiments in which these factors may be operative are obvious and somewhat disturbing. Herein lies a part of the importance of this work; the factors which are demonstrated here may affect certain types of experiments which have no primary relationship to this field, for these influences may be operative in any experiment which involves the transfer of living cells from one host to another.

An inspection of figure 1 reveals that by using the same mouse and the same tumor, high resistance or almost no resistance may be obtained according to the blood used. Or, if the tumor and the immunizing blood are kept constant, the response varies with the host. Again, if the host and blood are kept constant, the response varies with two different tumors. The degree of response elicited by a given blood does not seem to be related to the susceptibility of the strain from which the blood was derived, but the data available do not provide sufficient evidence for a decision as to possible intrinsic qualities of the blood. From these results, however, one might say, upon limited evidence, that when the blood is closely related to the tumor but not to the host, a high degree of resistance will be attained.

Considering the results obtained with the Bashford tumor, there is a reduced incidence of takes in both strains given either blood, but for either strain the reduction is greater when the alternate blood is used as the immunizing agent than when the corresponding blood is given. This relation is also true in the

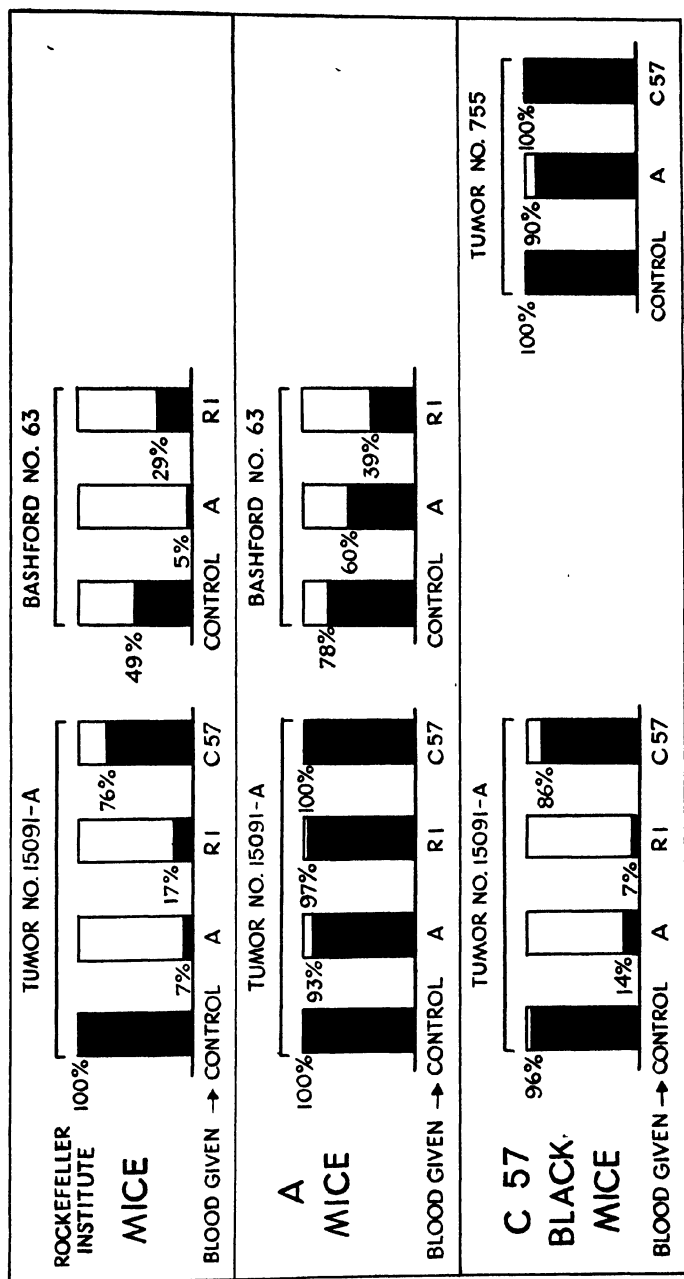


FIG. 1.—Summary of results in all mice. Individual columns represent all mice in a category. Shaded portions represent susceptible animals, and clear portions resistant animals. Percentages are shown at the left of each column. The groupings show difference of host or tumor. All mice in the first row are of Rockefeller Institute strain; in the second row, A strain; and in the third row, C57 Black strain.

group of C57 Black mice inoculated with tumor 15091-A. It does not hold in the case of Rockefeller Institute mice inoculated with tumor 15091-A, where C57 Black blood conferred least resistance. For lack of a better explanation this last will have to be ascribed to variability in the animal stocks and treated as an exception until more evidence is obtained.

These relations favor the belief that the mechanism involved is a sensitization type of phenomenon, in the sense suggested above, and depends upon the foreignness of the tissues used. If one accepts this viewpoint, the results are confirmatory since it seems obvious that the cells (i. e., the blood cells) of the alternate strain are more foreign to any host than are the cells of the corresponding strain, and the cells of the tumors in each of these cases are foreign to the host. Further support is given to this viewpoint by the failure to induce resistance in either the A strain or the C57 Black strain against a tumor of homologous origin. This result is but a step removed from Haaland's reported failure to induce resistance against an autograft.

Such a line of reasoning leads to the thought that, as the foreignness of the immunizing dose toward the host is decreased, there should follow a corresponding decrease in the degree of resistance induced. The final step in this series is, of course, the injection of autologous cells, which is known to be without effect. The data here may present an intermediate step since the relative reduction in the percentage of takes produced by giving a mouse blood from an individual of its own strain is less in the case of the more inbred, and supposedly more homogeneous, strains (i. e., Little's A and C57 Black).

Thus the evidence obtained by these experiments together with that of previous workers gives force to the most important implication of this work, which is—if the considerations above are correct—that the search for a means of treating human cancer based upon these principles (e. g., autoplasmic grafting, sensitizing injections, etc.) is likely to prove a fruitless one.

Many unknown factors are involved in these relationships, and the final solution of the problem waits upon more data. Further work along these lines is being undertaken.

SUMMARY

It has been shown that when resistance to transplantable tumors is induced in susceptible mice by subcutaneous injection of defibrinated whole blood, the degree of resistance varies with the genetic interrelationships of the host, the tumor, and the donor (of the blood).

With the two pure-line tumors used, no significant resistance could be induced in an inbred mouse against a tumor derived from the same strain.

The implications of these findings are discussed.

Acknowledgment is due Dr. James B. Murphy, in whose laboratory this work was done, for helpful cooperation.

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DIPLOID AND ANDROGENETIC HAPLOID HYBRIDIZATION BETWEEN TWO FORMS OF RANA PIPIENS, SCHREBER¹

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INTRODUCTION

One of the better methods for examining nucleo-cytoplasmic relationships is to combine identical nuclei with different cytoplasm. This can be achieved in a number of ways. For example, if the gametes of two different species are brought together to form reciprocal diploid hybrids, it is expected that at least in the early stages such hybrids will have identical nuclei and different cytoplasm. This arises from the fact that in most cases the maternal parent contributes practically all of the cytoplasm. Differences which may appear in the development and heredity of the reciprocals can therefore be related to differences in the egg cytoplasm of the two parent forms. If such cytoplasmic differences are observable and measurable, the possibilities are obvious.

The same end is achieved by combining the male nucleus of one species, subspecies or race with the nucleus-free egg cytoplasm of the same species and another species, subspecies or race. If the androgenetic or merogonic² homospemic haploid and heterospemic haploid resulting from this procedure show dissimilarities, these must be related to cytoplasmic differences.

It is scarcely necessary to point out that the results of such procedures seldom if ever satisfy the preconceived possibilities. The results from reciprocal diploid hybrids may be limited by an incompatibility of combined nuclei or nuclei and cytoplasm; or, where this is not the case, by the absence of sufficient cytoplasmic difference to produce an effect. And heterospemic haploids are usually less satisfactory. In the best of circumstances haploid organisms develop poorly and can only be produced in a limited group of materials. Apparently as a result of a high degree of incompatibility between nucleus and cytoplasm, the development of most androgenetic species hybrids is extremely abnormal and ceases in the earliest stages.

¹ Data obtained, in part, from experiments performed during tenure of National Research Fellowship at Princeton University.

² The term androgenetic refers to the development of the *whole* egg with only the male nucleus functional; merogonic refers to the similar development of an egg-fragment (Wilson, 1925).

Despite such results, the possibility remains that hybridizations with heretofore untried material such as the North American Salientia may reveal one or more compatible combinations with the desired qualities. Experiments to test this possibility have been made and the following pages report one such investigation. Two distinct but closely related forms of the genus *Rana* have been combined reciprocally to form diploid and androgenetic haploid hybrids. The results form an interesting addition to the existing data on nucleocytoplasmic relationships.

The author is very grateful to Prof. G. Fankhauser for helpful suggestions and criticisms.

Materials

The gametes for these hybridization experiments were derived from two distinct forms of frog, one collected from the meadows of northern Vermont, the other from the immediate vicinity of Philadelphia. Both forms are commonly referred to as *Rana pipiens* and possibly represent different races or subspecies of that species. More attention will be given to their probable relationship in the discussion.

That the two forms are distinct is indicated by their general characteristics (Figs. 1 and 2), and also by the results of these experiments. These same features also indicate that the two are closely related. Therefore, as a temporary assumption and to facilitate the description of the experiments, the frogs are being considered as northern and southern forms or races of the same species. As such they will be referred to in the succeeding pages of this report.

A brief description, supplemented by Figs. 1 and 2, will indicate their major differences and similarities.

The northern form (from northern Vermont) is generally larger and, relative to its body size and weight, it has shorter jumping legs than the southern form. The head is obtuse; the vocal sacs on the male are less apparent; the dorso-lateral folds are broad; the skin is thick; the palmation is full. Distinctive features of pigmentation include spots that are larger and surrounded by a green or yellow border; the cross-bars on the tibia are generally complete; the posterior border of the thigh is marked by black spots on a continuous white background; the tympanum does not show a central light spot with the same clarity as in the southern form.

The southern form (from the vicinity of Philadelphia) is generally smaller and, relative to its body size and weight, its legs are longer. The head is more acuminate; the vocal sacs are thin-walled and usually apparent; the dorso-lateral folds are narrow; the skin is thin; the palmation is shallow. Distinctive features of pigmentation include spots that are smaller and, under the same laboratory conditions as the northern form, not surrounded by clear borders; the cross-bar markings on the tibia are generally interrupted along the dorso-



FIG. 1. Photographs of representatives of northern (left) and southern (right) forms of *R. pipiens* used in these experiments.



FIG. 2. Photographs showing pigmentation of jumping legs, northern (left) and southern (right).

lateral surface; the posterior surface of the thigh is marked by white spots on a continuous black background; and the tympanum generally shows a central light spot.

Methods

The eggs were obtained in every case from frogs which had been induced to ovulate by frog pituitary injections. As much care as possible was taken to avoid removing the eggs in the immature or over-ripe condition. The sperm for insemination were obtained by macerating the testes in 10 per cent Ringer's solution and every precaution was taken against contamination of one suspension with sperm from another.

The eggs of each frog were inseminated in two batches, the first with sperm of the same form, the second with sperm of the other form. Thus, in all, four batches of eggs were inseminated. An interval of 15 to 20 minutes was allowed to elapse between each insemination to provide time for removing the egg pronucleus from a number of eggs of each batch. By this procedure 8 different types of embryos were produced. These are listed below with the designation used for each in the balance of this report.

| | |
|----------------------------------------------------------------------------------------------|--------------------------|
| Homospermic diploids of the northern form | <i>n</i> |
| Homospermic haploids of the northern form | <i>n</i> /2 |
| Heterospermic (hybrid) diploids from eggs of northern form and sperm of southern form | <i>ns</i> |
| Heterospermic (hybrid) haploids from cytoplasm of northern form and nucleus of southern form | (<i>n</i>) <i>s</i> /2 |
| Homospermic diploids of the southern form | <i>s</i> |
| Homospermic haploids of the southern form | <i>s</i> /2 |
| Heterospermic (hybrid) diploids from eggs of southern form and sperm of northern form | <i>sn</i> |
| Heterospermic (hybrid) haploids from cytoplasm of southern form and nucleus of northern form | (<i>s</i>) <i>n</i> /2 |

The egg pronucleus was removed with a fine glass needle as described in a previous report (Porter, 1939). Adequate numbers of pure and hybrid haploids were thus easily prepared (Table I).

All embryos were kept under identical conditions of temperature (19.4°C.) and space. In fixation of representative forms for a permanent record, a mercuric chloride, acetic acid, and formaldehyde mixture was generally used. The same sequence and time intervals were observed in fixation as had been observed in fertilization. Thus it was assured that all animals fixed at the end of a period of time were of the same age.

RESULTS

The description which follows is based upon observations made in the experiments listed in Table I. The possibility that the same results could occur

by coincidence in all four series of crosses is slight if not negligible. The analysis is confined to such characteristics as were apparent from external examination and only those characteristics which were uniformly shown by the animals in all four groups are stressed in the succeeding paragraphs.

TABLE I³

| Exp. | Date | Number of homo-spermic haploids produced | Number of hetero-spermic (hybrid) haploids produced | Treatment |
|------|---------------|------------------------------------------|------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 1. | Jan. 9, 1939 | 23 <i>n</i> /2 21 <i>s</i> /2 | 41 (<i>n</i>) <i>s</i> /2 35 (<i>s</i>) <i>n</i> /2 | Preliminary comparison of living animals made throughout development. Representative embryos fixed at end of 3, 5, 7, 9, 10, and 11 days. |
| 2. | Jan. 17, 1939 | 29 <i>n</i> /2 37 <i>s</i> /2 | 30 (<i>n</i>) <i>s</i> /2 44 (<i>s</i>) <i>n</i> /2 | Living animals compared throughout development. Representative forms fixed at end of 2, 3, 4, 5, 6, 7, and 8 days. Diploid hybrids and controls carried through metamorphosis for examination of inheritance. |
| 3a. | Feb. 15, 1939 | 37 <i>n</i> /2 24 <i>s</i> /2 | 48 (<i>n</i>) <i>s</i> /2 43 (<i>s</i>) <i>n</i> /2 | Living animals compared throughout development. Special attention given to gastrulation and neural tube formation. Representative forms fixed at end of 36, 43, 48, 51, 53, 55, 57, 59, and 61 hours and at 3 and 4 days. |
| 3b. | Feb. 15, 1939 | 21 <i>n</i> /2 45 <i>s</i> /2 | 36 (<i>n</i>) <i>s</i> /2 41 (<i>s</i>) <i>n</i> /2 | Living animals compared. Special attention given to study of older stages. Material fixed at end of 32, 54, and 60 hours and 3, 5, 6, 8, 9, and 10 days. |

³ The same females were used as a source of eggs for experiments 3a and 3b. Otherwise, different parents were used in each cross.

For greater clarity the description of the 3-day-old embryos is presented first. With the differences of these in mind the descriptions of the younger and older stages have greater meaning.

Three-Day-Old Embryos

The following account is illustrated by the outline drawings in Fig. 3 and to them reference is constantly made.

The homospermic (control) diploids of the two races develop at approximately the same rate at 19.4°C. and, stage for stage, are comparable at the end of 72 hours. The differences, though real, are very slight and were clearly recog-

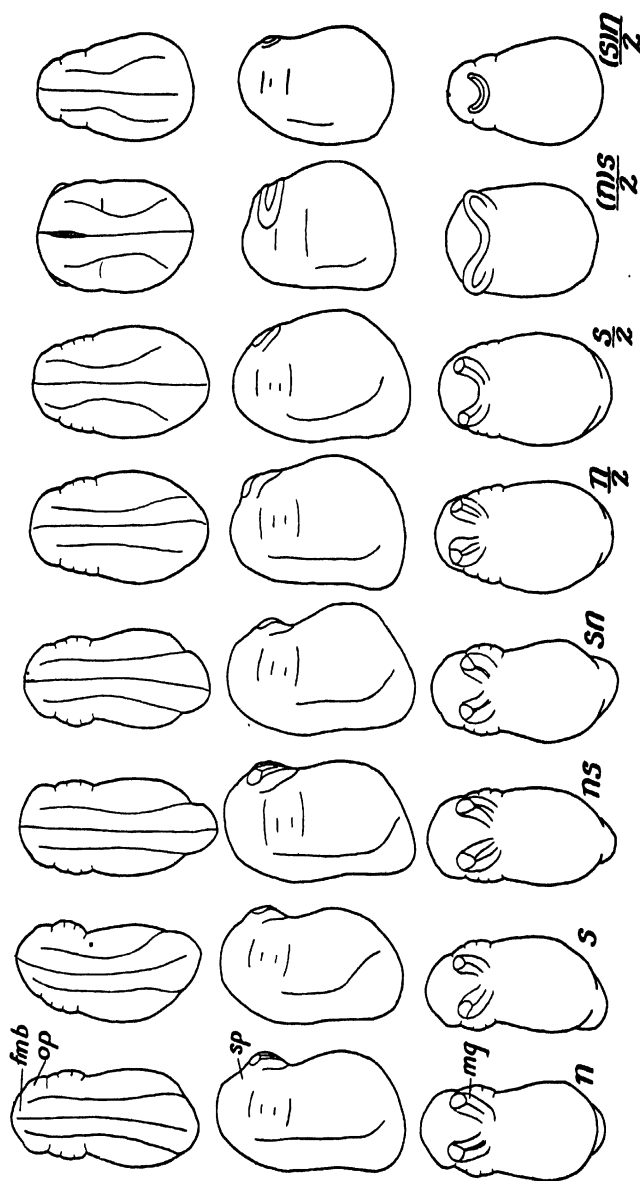


FIG. 3. Outline camera lucida drawings of 72-hr old embryos. From left to right the drawings represent: *n*, homospermic diploid control of northern form; *s*, homospermic diploid control of southern form; *ns*, heterospermic haploid from northern egg and southern sperm; *sn*, heterospermic diploid from southern egg and northern sperm; *n/2*, heterospermic haploid control of northern form; *s/2*, homospermic haploid control of southern form; *(n)s/2*, heterospermic haploid (androgenetic hybrid) from northern cytoplasm and southern sperm; *(s)n/2*, heterospermic haploid from southern cytoplasm and northern sperm. Magnification, 12.5 X. The same sequence and magnification are observed in Figs. 4, 5, 6, and 7.

It can be seen that head structures (fore- and mid-brain regions, *fmb*, optic vesicle outpocketings, *op*, sense plate, *sp*, and mucous gland, *mg*) are larger in *n*, *ns*, *n/2*, and *(n)s/2*, than in *s*, *sn*, *s/2*, and *(s)n/2* respectively. The tail-bud, *t*, tends to be larger in the latter. These differences are clear between the reciprocal heterospermic diploids, *ns* and *sn*, and pronounced between the heterospermic haploids, *(n)s/2* and *(s)n/2*.

nized only after repeated examination of material available. The northern diploids compared with the southern diploids show larger gill plates, a larger sense plate and larger mucous glands. Relative to body size the head of n is the larger. The neural tube is broader and stands up more distinctly in n . The tail-bud in n is smaller and directed more dorsally than in s , thus creating a deeper depression in the back of n . In relation to head size, the abdomen of s is larger than that of n . To these differences it can be added that the head flexure dorsal to the posterior margin of the gill plate is more pronounced in s than in n .

The homospermic (control) haploids of the two races, as is normal for haploids, are retarded in their development. Compared with each other they show in an exaggerated form the same differences that were given for the diploid controls.

The heterospermic (hybrid) diploids show approximately the same rate of development as the homospermic diploids and as each other. They differ in body proportions and show in accentuation the differences which are difficult to see between the pure diploids of the two forms. A greater proportion of ns consists of head structures than in the reciprocal hybrid. Conversely, a greater proportion of sn consists of abdomen and tail-bud. The mucous glands and sense plate are larger in ns and, posterior to the medulla, ns shows a smaller neural tube which terminates in a smaller and more dorsally directed tail-bud.⁴

The heterospermic (hybrid) haploids show in most exaggerated form the differences which have been referred to as existing between control diploids and haploids and more distinctly between the hybrid diploids. It is readily apparent that oral suckers, gill plates, and sense plate are greatly enlarged in $(n)s/2$. Relative to head size, the abdomen and tail-bud of $(s)n/2$ are much larger than the same structures of $(n)s/2$. It can be further noted that the head of $(s)n/2$ is flexed more ventrally than $(n)s/2$ and the back of the latter is convex while in the former it is concave. These differences are the expression of the decidedly dissimilar embryology of the two reciprocal heterospermic haploids and not a difference in age or stage.

Summary.—In general the combinations which include cytoplasm of the northern form are characterized by larger head primordia and smaller posterior axial structures than are observed in those with southern cytoplasm. Such differences, only slightly apparent in the pure diploid controls, become progressively more accentuated in the homospermic haploids, the heterospermic diploids and in the heterospermic haploids.

It is of interest to observe now the earlier and later expressions of these

⁴ If these experiments had been confined to the production and study of diploid hybrids, it is doubtful if the differences would have been considered great enough to warrant any conclusions. Supported by the evidence from androgenetic hybrids, however, the significance of the differences is unquestionable.

general differences as shown by an examination of the earlier and later stages in the ontogeny of the various combinations.

Neural Tube Formation

The description under this heading is derived from a comparative study of living material and of representative embryos of the eight different types fixed at intervals of two hours from 51 to 61 hours after insemination. Illustration is provided by outline figures 4, 5, and 6 which are respectively representative of developmental stages reached at the end of 55, 59, and 61 hours.

The homospermic diploids, during this period, are very similar both in character and rate of development. As the neural plate is outlined, it becomes apparent that its anterior portion plus the sense plate are larger in *n* than in *s*. These differences increase in clarity as the neural folds are elevated and gill plates appear (Figs. 5 and 6). At this latter stage, *s* flattens dorsally and shows a greater elongation of that portion of the neural groove posterior to the gill plates. At the same time the neural plate and folds are more distinctly elevated in *s*. Although the neural plate and folds may be outlined in *s* slightly in advance of *n*, the closure of the folds is more rapid in the latter. During neurulation the blastopore of *s* is bounded laterally by distinctly thickened lips.

The homospermic haploids show in exaggerated form the slight differences existing between the diploid controls. In equivalent stages (55 hours) the neural folds of *n/2* are thicker, the sense plate and other primordial head structures are larger than in *s/2*, whereas the latter shows a greater elongation of the neural plate, especially that portion of it determined to be spinal cord. In *s/2* the neural folds show a greater elevation, the dorsal surface straightens or flattens out, and pronounced lateral lips bound the blastopore. Besides stage-for-stage structural differences, the differences in rates of separate morphogenetic processes are also accentuated. For example, it is noted that *s/2* completes gastrulation considerably ahead of *n/2* and only slightly after *s*, whereas in *n/2* the neural folds appear to close slightly before they do in *s/2*.

The heterospermic diploids show clear-cut differences. Compared with each other in the early stages of neurulation, it is apparent that the *ns* forms have a shorter neural plate which is abnormally broad at the anterior end. The reciprocal *sn*, on the other hand, has a long and narrow neural plate. As the two differ from each other so do they differ from their maternal control diploids though to a lesser degree. Other features of dissimilarity include neural folds which are larger in *ns* than in *sn* and which are more distinctly elevated in *ns* than in the control diploid, *n*. In this latter respect they approximate the condition noted above as apparent in the paternal diploid control. As neural tube formation continues, the greater size of the head primordia and the shorter neural plate and groove are maintained in *ns*. Though the neural plate and folds are outlined almost simultaneously in these

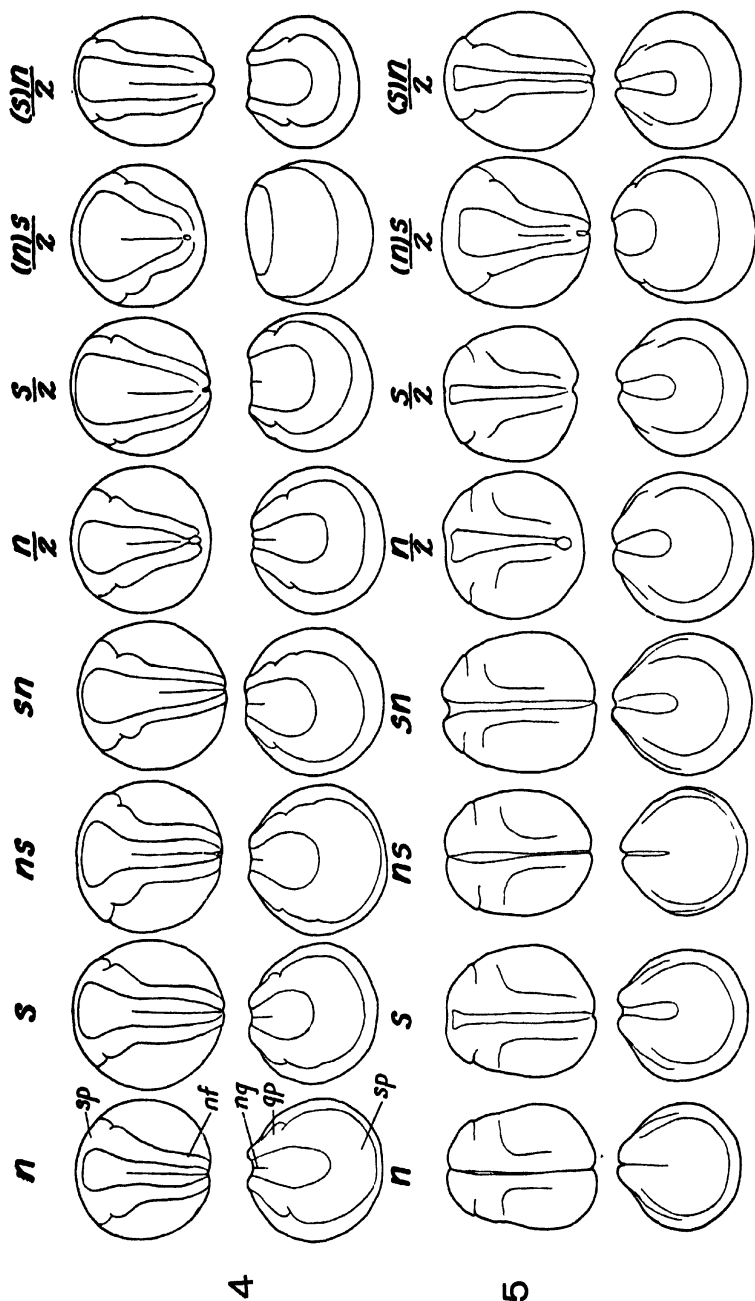


FIG. 4. Dorsal and anterior views of 55-hour-old embryos. It can be noted that, relative to size of abdomen, the head primordia (sense plate, sp , gill plate, gp) of n , ns , $n/2$, and $(n)s/2$ are larger than the same of s , sn , $s/2$, and $(s)n/2$. Neural folds, nf . Neural groove, ng .

FIG. 5. Dorsal and anterior views of 59-hour-old embryos.

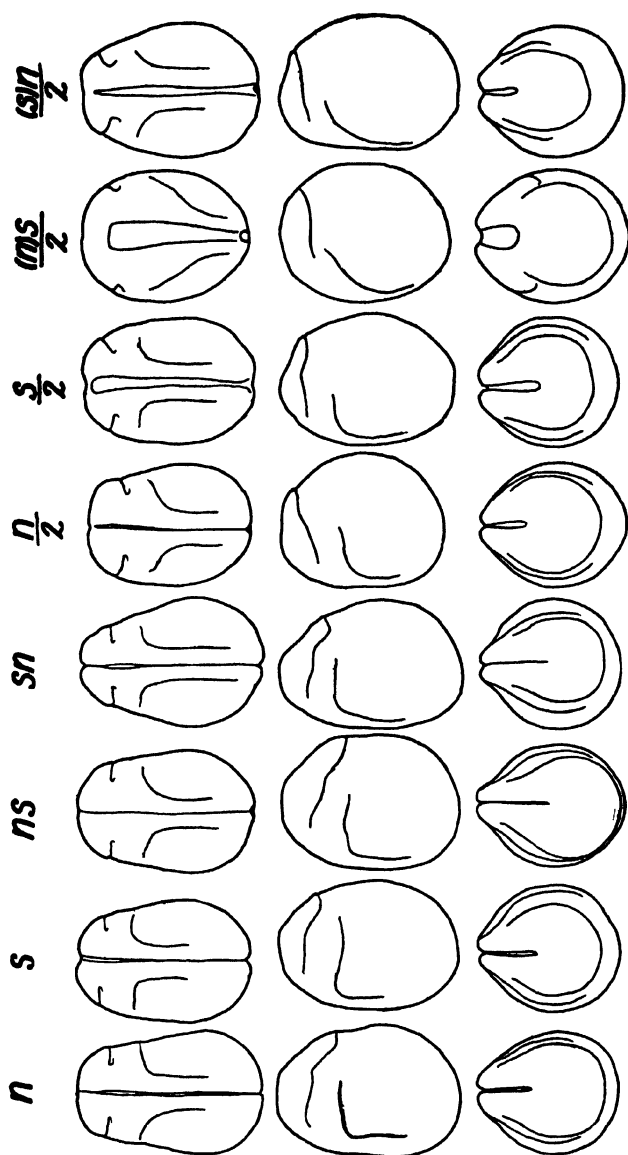


FIG. 6. Dorsal and anterior views of 61-hour-old embryos

reciprocal hybrids, the folds come together in *ns* slightly before they do in *sn*. The lateral borders of the blastopore are swollen in *sn* to form lips as in *s* and *s/2*.

The *heterospermic haploids* show very striking differences during the development of the neural tube. At the end of 51 hours (not illustrated) (*s*)*n/2* has a dorsal flattened surface, abnormally straight from anterior to posterior ends. The neural plate is clearly outlined and is very narrow. Gastrulation has been completed, and there are extremely pronounced lips on both sides of the blastopore. Contrasting with this, the reciprocal (*n*)*s/2* is considerably retarded. The yolk plug is still apparent and the limits of the neural plate are not visible. The (*n*)*s/2* embryos are flattened dorso-ventrally and present a large, swollen appearance. By the end of 55 hours the neural plate of (*n*)*s/2* has been outlined. It is as broad as it is long and that portion designated to become neural tube is extremely short. The yolk plug persists. At this same time in (*s*)*n/2* the neural plate has lengthened and the neural folds have approximated to some extent. At 59 hours (*n*)*s/2* continues to show a short, broad neural plate, bounded by prominently elevated neural folds. This latter feature is a characteristic of *s* embryos and its appearance in these (*n*)*s/2* embryos represents the appearance of a specific paternal character. In the reciprocal it is not shown. It is a feature which will be easier of description and analysis when sectioned material is available. By 61 hours the neural folds of (*n*)*s/2* have started to approach and subsequent observations have shown that once started this process proceeds more rapidly here than in (*s*)*n/2*. At this time and later there is little elongation of the neural plate in (*n*)*s/2* and the yolk plug still persists in some cases. The sense plate and gill plates are abnormally large. The structure of the reciprocal hybrid (*s*)*n/2* at 61 hours is characterized by neural folds about ready to close, an elongate neural tube, and extremely small head primordia which foreshadow the diminutive head size of later stages.

Summary.—In summarizing, a few generalizations can be made. Those combinations which include cytoplasm of the northern race, including the diploid controls, are characterized by: (a) neural plates which when outlined tend to be shorter, and broader anteriorly, and (b) head primordia which are larger. The reciprocal combinations with cytoplasm of the southern race are, on the other hand, characterized by: (a) longer and narrower neural plates, (b) smaller head primordia, and (c) pronounced lateral lips on the blastopore. These differences become increasingly apparent as one compares respectively the diploid controls, the haploid controls, the reciprocal hybrid diploids, and the reciprocal androgenetic hybrids.

At one stage in the development of the neural folds it is apparent that they are more sharply delimited and distinctly elevated in the diploid of the southern form. This characteristic is repeated in the hybrid diploids and in the andro-

genetic hybrids containing the southern nucleus. It seems to represent, therefore, an inheritable embryonic characteristic capable of expressing itself in the foreign cytoplasm of the northern race. More careful analysis of this phenomenon is needed.

There are also to be noted slight differences in the times of occurrence and rates of the same morphogenetic processes. Relative to blastopore closure the neural plate is outlined earlier in those combinations with northern cytoplasm. Relative to time after fertilization, however, this may be later. Once clearly outlined the neural folds of the combinations with the northern cytoplasm seem to close more rapidly.

Gastrulation

This phase of the embryology of these various combinations was studied from living material and from representative forms fixed at the end of 36, 43, and 48 hours. A few differences between the gastrulae of those forms with northern cytoplasm and those with southern cytoplasm occur consistently (excepting the diploid controls where they are not sufficiently pronounced to be clearly evident) and become progressively more pronounced in haploid controls, heterospermic diploids, and heterospermic haploids. Those combinations with the cytoplasm of the southern race show a larger gastrular angle, a smaller completed blastopore, epiboly largely from the dorsal and lateral borders of the blastopore, and toward the end of gastrulation, an increasing thickening of the lateral blastopore lips. Those combinations with northern cytoplasm show a smaller gastrular angle, a larger blastopore, epiboly from all sides of the blastopore, and thin blastopore lips. Gastrulation appears to begin earlier in s , $s/2$, $(n)s/2$, and simultaneously in sn and ns . Observations recorded on this feature and on the rate of gastrulation are not sufficiently extensive to be conclusive.

It would seem that the greater gastrular angle and the greater epiboly of the dorsal lip in those haploid and hybrid embryos with the southern cytoplasm are the early abnormalities related to the longer neural plate of later stages. It also appears that the thickened lateral blastopore lips of these same forms are the early expression of the larger tail-bud and somites of later stages (Bijtel, 1931). The opposites of these same features in those forms with the northern cytoplasm are probably related to the shorter neural plate and smaller tail-buds of their later stages.

Older Stages (4-10 Days)

The studies reported in this paper have been largely devoted to the younger stages hence only the most general features of the older stages will be described under this heading. Reference should be made to Fig. 7.

The homospermic diploids of 4 and 5 days continue to show the slight differ-

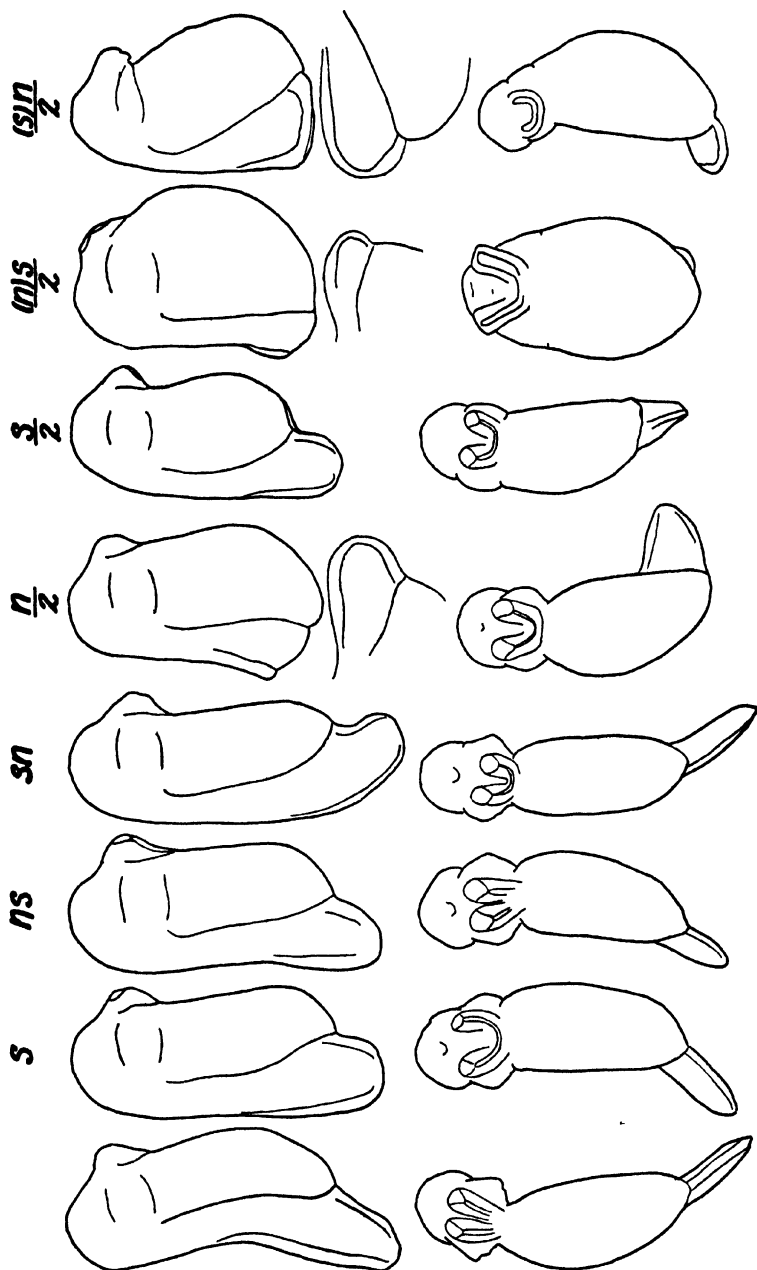


FIG. 7. Lateral and ventral views of 4-day-old embryos. It can be noted that early differences in relative body proportions persist in the various combinations.

ences which existed between the 3-day-old embryos. In the older stages, however, these differences become increasingly subtle. Relative to body proportions, the head of n remains larger while the tail of s is more elongate and larger in relation to the rest of the embryo. The dorsal concavity of n persists in greater prominence than in s .

The *homospermic haploids* differ in the older stages, as they had in the earlier stages, in relative size of body parts. The differences are similar to but more distinct than those occurring between the diploid controls.

The *heterospermic diploids* demonstrate more clearly the perpetuation of early differences. The combination, ns , persists in showing at various ages a larger head with larger mucous glands and a smaller dorsally directed tail. The converse of these features are shown by the reciprocal. Such differences are retained into the later stages of development, especially the relative head and tail size. Clear-cut appearance of paternal characteristics is recognized first in stages showing chromatophore patterns.

The *heterospermic haploids*, as in the younger stages, show the most striking differences. It is recognized, however, that these differences are less pronounced in the older stages suggesting some regulation. It is easily noted that the head of $(n)s/2$ and its component structures remains larger and the tail remains smaller and directed dorsally. The androgenetic hybrid, $(s)n/2$, on the other hand, is characterized by a small head and a ventrally directed tail which is referred to as larger because of the broad heavy somite mass at its base. These features were foreshadowed in the early embryonic development.

Summary.—Those combinations with cytoplasm of the southern race tend to have smaller head structures and, relative to body size, larger tails than the reciprocals with northern cytoplasm. Such features are doubtless the expression of earlier embryonic differences in the size of head primordia and tail-buds.

Survival of Various Combinations

Since representative embryos were sacrificed for fixation at various intervals, no definite data can be given to demonstrate survival value. Nevertheless, the observations made permit the following statements.

The heterospermic hybrids, in the majority of cases, develop up to and through metamorphosis. Beyond that stage no data are available.

The homospermic haploids of the two races demonstrate approximately the same viability. They continue their development, on the average, for from 8 to 12 days up to approximately stage 24 (Shumway, 1940).

The *heterospermic haploids* develop through the early stages showing only a small percentage of deaths. About one-fourth to one-third fail to hatch and of those which hatch the majority live for from 7 to 11 days or up to stages 22 and 23 (Shumway, 1940). They are slightly less viable than the haploid controls. In a small percentage of cases a gill circulation is established and in

scattered cases growth continues sufficiently long to show the first guanophores. No differences in viability were recorded as existing between the two reciprocal combinations.

Identification of Haploids

The haploids were identified as such solely on the basis of the type of development. In an earlier study (Porter, 1939) it was shown that embryos which arise from operated eggs can be expected to develop as haploids in 90 per cent of the cases. Furthermore, such haploids were found to show certain definite characteristics when compared with their diploid controls. Hence, in these experiments, it has not been considered necessary to make a complete cytological examination of every embryo which developed from an operated egg, especially since group characteristics rather than individual characteristics have been considered. The isolated cases of diploidy which did appear among the embryos from operated eggs were readily identified by their development, cell size, etc.

In order to establish the chromosome count of the southern form, the tail-tips of several haploids were examined. The examinations made indicated 13 to be the haploid count. This is the same as for the northern form determined in an earlier study.

Other Observations

In the case of two of the above-described experiments, surplus diploid embryos both pure and hybrid were kept for examination as older tadpoles and as metamorphosed frogs. The pure diploids showed differences characteristic of the northern and southern forms; the hybrid diploids showed blended inheritance with indications of stronger paternal influence in certain features of pigmentation. Thus despite the blending the reciprocal hybrids were distinguishable. These observations indicate that at least the differences in pigmentation between the two parent forms are related to differences in nuclear factors.

Two further observations, which, because of the small amount of evidence supporting them, must be considered as very preliminary, are briefly described. They are presented because of their interest as possible leads for experiments aimed at determining the nature of the factors responsible for the peculiar development of the hybrid embryos described above.

Since the frogs used were obtained from widely separated northern and southern points, it was considered of interest to examine the effect of high temperatures. Embryos representative of the 8 different combinations treated above were placed in a warming oven at 28°-29°C. Those combinations with cytoplasm of the southern form were not apparently damaged and developed in the typical manner, whereas those combinations with northern cytoplasm

were markedly affected and only a very small percentage of the original number developed through 6 days. Controls kept at 19.4°C. developed normally.

Cytological examination of a few pure diploid 3-day-old embryos revealed some interesting differences in nuclear size and size of yolk granules. Measurements of nuclei of identical tissues of the two forms showed those of the northern to be the smaller. Measurements of the yolk granules revealed those of the northern to be much the larger.

DISCUSSION

The discussion which follows will be confined to a consideration of the probable relationship of the animals used and to the more general aspects of the cytoplasmic and nuclear influences demonstrated. A detailed and inclusive treatment must await the accumulation of data from a more thorough study of these and similar hybrids. In a sense, then, this constitutes a preliminary report.

When the experiments were first undertaken the parents were thought to represent two distinct species. This conclusion was based on differences which the animals showed and also on the authority of amphibian taxonomists (Kauffeld, 1937; Stejneger and Barbour, 1939). An examination of the literature soon revealed, however, that considerable confusion exists in the classification of the leopard frogs or frogs of this type resident in the eastern states and possibly over a wider area. Differences between those forms resident in northeastern and those in the southeastern states have long been recognized, but it appears that sufficient material from a variety of localities has never been examined to make a conclusive analysis of the species. A brief reference to the writings of a few authorities on Salientia classification will serve to illustrate this confusion.

It should be recalled that the southern forms used in these experiments were collected in the vicinity of Philadelphia and the northern forms in northern Vermont. From its place of collection, the southern form doubtless coincides in appearance with that type early described by Schreber (1782) as *Rana pipiens* (Kauffeld, 1936 and 1937). Later, Cope (1889), from examination of forms collected in a variety of localities, chose to describe the leopard frogs under three subspecies. The southernmost type he called *Rana virescens sphenocephala*; the type from the Atlantic coast *Rana virescens virescens* (probably same as Schreber's *R. pipiens* and the Philadelphia type of this study); and the type of northern distribution he called *Rana virescens brachycephala* (his description of which coincides perfectly with the northern form used in these experiments). More recent authors (Wright, 1933; Dickerson, 1906) have pictured and described the northern form as the typical *R. pipiens* and both it and the southern form have been considered as such by teachers and investigators alike. Most recently Cope's nomenclature has in part been revived,

only instead of using a subspecies classification, the three types have been placed in separate species. Thus the most southern form is called *R. sphenocephala*, the Philadelphia form falls within the range of *R. pipiens* and the northern becomes *R. brachycephala* (Kauffeld, *loc. cit.*, and Stejneger and Barbour, *loc. cit.*).⁵ It was on the basis of this latter classification that the frogs were originally considered to represent two species, *R. pipiens* and *R. brachycephala*. It is clear, however, that this classification is uncertain and consideration of some further points increases this uncertainty.

In the first place, it would seem that the two forms hybridize too successfully to be representatives of two distinct species. It is true that a few distinct species of the Salientia have been successfully hybridized (Born, 1883; Pflüger and Smith, 1883; Héron-Royer, 1891; Montalenti, 1933; Dürken, 1938 and Moore, 1940) so that the successful crossing of these two forms, even if they represent distinct species, is not without parallel. What is unique is the result of androgenetic hybridization, for no case involving the Salientia has been reported in which the development of an androgenetic or merogonic species hybrid continued to the advanced stages obtained in these experiments.⁶ In other words, the compatibility of the two forms is greater than would be expected of two distinct species.

In the second place, it can be said that the characteristics of the two forms do not differ sufficiently to place them in separate species. Aside from body proportions, which is dealt with below, the major difference is one of pigmentation. This difference, it can be noted, does not involve the pattern but chiefly the size of the markings and intensity of the coloration. These are features which in other animals may vary considerably among races.

Finally, recalling that the two forms were collected from different northerly and southerly climates, and considering the points about to be discussed, the differences in body proportions likewise do not support a species relationship. Taxonomists have long been acquainted with certain generalizations known as the Bergmann and Allen rules pertaining to differences in size and body proportions which can be recognized between the northern and southern races of warm-blooded species. The former of these states that northern races are larger; the latter, that the southern races have relatively longer body projec-

⁵ In footnote, Stejneger and Barbour (1939) indicate that the whole *sphenocephala*-*pipiens*-*brachycephala* complex needs further examination and possible revision.

⁶ Baltzer (1920 and 1933) reports that from the combination of *Triton taeniatus* cytoplasm and *Triton palmatus* nucleus heterospermic haploids develop to stages showing good eye formation, pigment, small branching gills, and pulsating heart. Though this represents advanced development as compared with the usual result with different Salientia species, the stage reached does not seem to be the equivalent of that reached by the best of the heterospermic haploids obtained with these two forms of *Rana pipiens*.

tions. Within recent years an increasing volume of research examining racial and subspecies differences has shown that characteristics other than body size and proportions may likewise vary in an orderly and predictable manner with a variety of environmental gradients. Inclusive surveys of these phenomena are to be found in the recent writings of Goldschmidt (1940), Rensch (1936) and others. But among the species of animals examined for chains of racial differences or "clines" (Huxley, 1938), it appears that species of Amphibia have been regrettably absent. Schmidt (1938) reviewed some measurements of species of Salientia and noted that relative to body size the leg length was greater for those representatives of a species which were collected from the more southern localities. The small number of animals examined and the preserved condition of these did not, however, permit any definite conclusions. Measurements of unselected groups of the two forms used in these crosses show the same tendency of the northern form to have a heavier and larger body structure relative to leg length.

It is possible that a thorough examination of the literature would reveal additional references to racial differences between frogs. For example, such differences are briefly mentioned in a paper by Pflüger and Smith (1883). Comparing the English race of *R. fusca* with the Königsburg race of the same species, they write:

"Der englische braune Grasfrosch ist etwas kleiner und schlanker als der deutsche, weniger stumpfschnauzig und von zarterer Haut."

The similarity between these differences and those noted between the Vermont and southeastern Pennsylvania forms of *R. pipiens* is obvious. This similarity takes on added interest when it is noted that roughly the same climatic differences (as indicated by mean annual temperatures) exist between East Prussia (44°F.) and England (50°F.) as between Vermont (43°F.) and southeastern Pennsylvania (52°F.).

In view of these observations and the fact that racial variations accompanying climatic gradients have been found in a great many species of both the animal and plant kingdoms, it seems probable that species of frog when thoroughly examined will likewise show various clines with regard to temperature and other environmental factors. In the meantime it can only be maintained that the two forms used in these crosses probably represent two races of the same species.

If such is the case, the results of these experiments are of interest in demonstrating that racial differences involving body proportions can be recognized in early embryonic stages, and that at least some of the factors responsible for these differences exist in the cytoplasmic organization of the egg (see below). This observation and others which will probably be made from a more extensive examination of these and similar crosses may prove of interest to students who concern themselves with factors involved in species formation.

Experiments examining the relative rôles of the nucleus and cytoplasm in heredity have generally shown the nucleus to be the sole bearer of factors controlling the appearance of specific adult and juvenile characteristics. Some of these experiments have combined the nucleus of one species with the cytoplasm of another to form merogonic hybrids, attempting thus to demonstrate the presence of hereditary units in the cytoplasm. Among these, the studies of the Hertwigs, Boveri, Baltzer, Hadorn, and Hörstadius are well known and frequently reviewed. With the possible exception of Hadorn's (1936) results, the demonstration of cytoplasmic inheritance has not been conclusive. The development of the merogonic hybrids generally ceases very early and even where it continues to a stage showing distinct species characteristics, as in certain sea-urchin merogons, the intermediate condition of the characteristic can be considered as an abnormality resulting from a degree of incompatibility between the nucleus and cytoplasm (Hörstadius, 1936). The early cessation of development which characterizes amphibian merogonic hybrids is probably also the result of a severe incompatibility.

It would appear that by using more closely related forms than those belonging to different species this problem of incompatibility could be overcome. To some extent this is probably true, but in using members of different races or subspecies, it is necessary to sacrifice the clear-cut distinctions which usually exist between the embryonic stages of different species and which are not to be expected between different races. Hence, the problem is fraught with difficulties and it is doubtful whether material such as used in these experiments, though it should be thoroughly examined, will supply any evidence in support of cytoplasmic-borne units of heredity even if present.

As distinct from heredity, cytoplasmic influence on development has been and can be demonstrated. This influence has been considered as the effect of plasmatic organization and composition upon the expression of nuclear factors. To this category of cytoplasmic activity the results of these crosses probably belong. Experimental embryologists have long recognized a high degree of cytoplasmic differentiation in a variety of eggs and the maintenance of such differentiation undisturbed is known to be essential in many cases for normal embryonic development. The cytoplasm of the egg by its organization, therefore, exerts an influence on the appearance of the adult in so far as this appearance is determined by the characteristics of the early developmental stages.⁷ Needless to say, the nature of this early cytoplasmic influence is not understood but every new demonstration of its presence offers new possibilities for its examination.

The consideration of the results of these experiments is facilitated if the development of the two control diploids is visualized as paralleling on opposite

⁷ In respect to even this cytoplasmic influence, it is to be remembered that considerable differentiation of the egg takes place in the presence of the maternal nucleus.

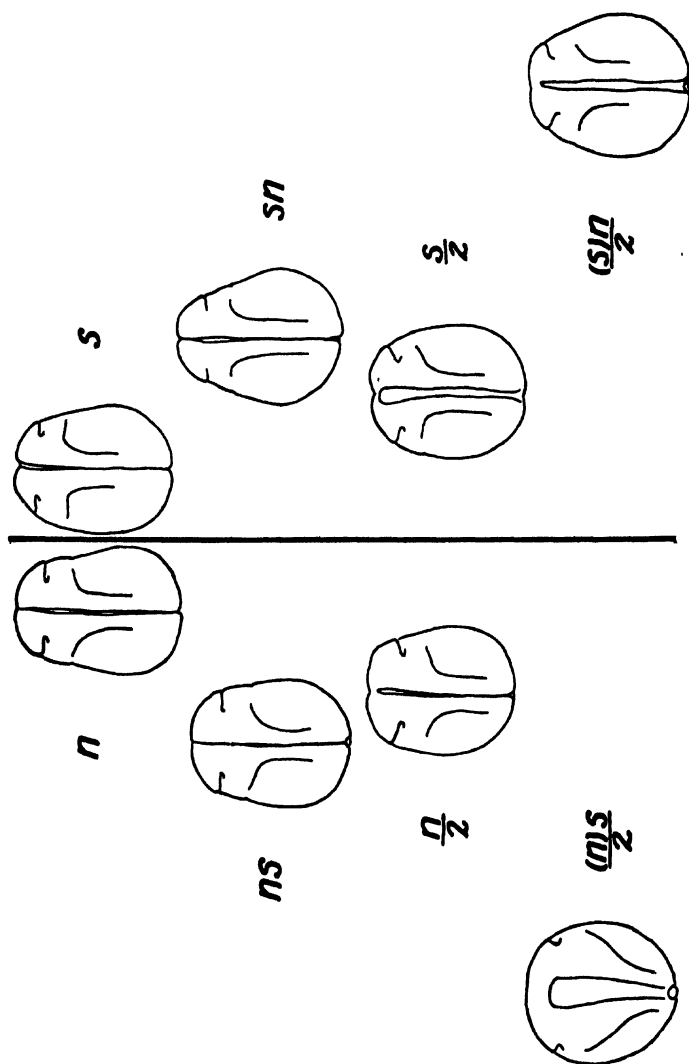


FIG. 8. Dorsal views of 61-hour-old embryos representing the development of the various nuclear-cytoplasmic combinations in relation to a hypothetical mean type, the central line. Those combinations with northern cytoplasm are on the left of the line; those with southern cytoplasm are on the right. The distance from the line represents the approximate degree of difference between the development of any one combination and the mean.

sides an average or mean type (Fig. 8). If the factors responsible for this slight departure from the mean are nuclear and the cytoplasm is perfectly neutral to nuclear control, then the diploid reciprocal hybrids would be expected to be identical and would in their development occupy a position coinciding with the hypothetical mean. Under the same conditions of nuclear control, the heterospermic haploid with the southern cytoplasm and northern nucleus would be expected to show the same development as the homospermic haploid of the northern form. Neither of these results is obtained. Instead, it is noted in the case of the reciprocal diploid hybrids that their development places them on opposite sides of the mean and at points more distant from the mean than their diploid controls. And in the case of the heterospermic haploids, the hybrid with the northern nucleus is not only further from the mean than the homospermic haploid of the northern form, but it is on the opposite side. Since the diploid hybrids can be considered as having identical nuclei and differing only in their cytoplasm, and since the same difference holds between the homo- and heterospermic haploids with nuclei of the same form, it follows that cytoplasmic influence is responsible for the dissimilarities existing between them.⁸ Therefore, the eggs of the northern and southern forms differ in some property or properties of their cytoplasm.

Are the nuclei identical or do they also differ? If the nuclei are considered as being identical and responding solely to cytoplasmic influence, then the development of the reciprocal diploid hybrids should parallel the mean at the same distance as their respective diploid controls. Or, under the same assumed conditions of identical nuclei, the heterospermic haploid with the southern cytoplasm should be identical in appearance with the homospermic southern haploid. Again, the results indicate that the assumed condition of identical nuclei cannot be valid. On the other hand, the intermediate position of the diploid control between the mean and the hybrid diploid with the same cytoplasm indicates that the nucleus of each race has compensating factors for the cytoplasm of that race. The same conclusion is also supported by the intermediate position of the homospermic haploid relative to the mean and the heterospermic haploid with the same cytoplasm. Evidently then, the nuclei of the two forms also differ and do so in such a way as to compensate in development for cytoplasmic differences.

Cytoplasmic and nuclear differences seemingly demonstrated, it is of interest to determine which is responsible for the slight dissimilarities between the control diploids, and the more distinct dissimilarities between the homospermic (control) haploids of the two races. It has been shown that each diploid control in its morphogenesis is on the same side of the mean as the hybrid diploid with the same cytoplasm, though not at the same distance. The homospermic haploids, in their development, parallel the mean at a greater

⁸ A heterozygous genome in the parent forms could not account for these differences.

distance than the control diploids, suggesting a lesser degree of compensation by the haploid nucleus. The homospermic haploid in its morphogenesis shows the same tendencies, though to a lesser degree, as the heterospermic haploid with the same cytoplasm. These facts suggest that the cytoplasmic differences are responsible for the slight dissimilarities between the diploid controls and homospermic haploid controls of the two forms. Further study may demonstrate whether or not these cytoplasmic differences are also related to the dissimilarities of the two adult parent forms.

What is the nature of these nuclear and cytoplasmic differences? There is not, of course, sufficient information available to answer this question. The presence of some degree of cytoplasmic organization in the amphibian egg has been shown to exist as early as 20 minutes after insemination (Fankhauser, 1930) and before first cleavage (Brachet, 1906), but the nature of this organization has not been demonstrated. Though the differences which are being

TABLE II

| Northern cytoplasm | Southern cytoplasm |
|---------------------------------------------------------|----------------------------------------------------------|
| 1. <i>Small</i> gastrular angle | 1. <i>Large</i> gastrular angle |
| 2. <i>Large</i> completed blastopore | 2. <i>Small</i> completed blastopore |
| 3. Neural plate abnormally <i>broad</i> at anterior end | 3. Neural plate abnormally <i>narrow</i> at anterior end |
| 4. Neural plate abnormally <i>short</i> | 4. Neural plate abnormally <i>long</i> |
| 5. <i>Small</i> tail-bud | 5. <i>Large</i> tail-bud |
| 6. <i>Large</i> head primordia | 6. <i>Small</i> head primordia |
| 7. <i>Small</i> abdomen relative to head size | 7. <i>Large</i> abdomen relative to head size |

examined cannot be described in precise terms, one feature of their relative nature does become apparent. It is clear from the results that some property or properties of the cytoplasm of the northern form tend to make the embryos with the cytoplasm of that form display certain features of development which, relative to the mean type representing normal development, are the exact opposite of those found in the embryos with the cytoplasm of the southern form (Table II). This infers that the differences in organization or composition, whether they be quantitative or qualitative, are of opposite natures as measured in terms of what they tend to produce in development. It has been noted further that the nuclei of the two forms have properties which tend to compensate for the cytoplasmic differences. Therefore the nuclei may also be considered to have properties of opposite nature. If this reasoning is correct, it seems that the nucleus of one form should supplement or enhance the cytoplasmic influence of the other form. This means that the development of the reciprocal heterospermic haploids should be sufficiently different to suggest the activity of something more than the cytoplasm. While there is no unit of measurement by which the degree of difference can be determined, it is clearly great (Fig. 8) and is probably contributed to by a nuclear influence.

The differences in size of yolk granules and nuclei which preliminary studies have shown to exist between the early embryonic stages of the two forms constitute the only concrete dissimilarities between cytoplasm and nuclei so far observed. What connection, if any, these may have with the actual nuclear and cytoplasmic differences responsible for the above results is not readily apparent.

It is of further interest to determine how these differences operate to produce the results described above. This point is brought into this discussion not because any definite answer can be provided but because certain experimental treatments which could be expected to alter the mode of operation of cytoplasmic and nuclear factors have produced similar results. For example, if a temperature gradient is applied to the developing frog egg, that portion at the warm end of the gradient develops abnormally large structural units (Huxley, 1927; Dean, Shaw, Tazelaar, 1928; Gilchrist, 1928, 1929, 1933). More specifically, if the gradient is applied "adjuvantly" (Huxley, 1927) along the animal-vegetal polar axis in blastula stages (i.e., with warm end of gradient at animal pole) the tail-bud embryos from a blastula so treated have slightly larger heads than the controls and those subjected to the reverse gradient (Huxley and Dean, Shaw, Tazelaar, *loc. cit.*). It is further reported by the same authors that an adjuvant gradient increases by several times the normal difference in size existing between animal and vegetal cells of the blastula stages. Gilchrist (1933) demonstrates that size differences of embryonic structures resulting from temperature gradient treatments are not due solely to age differences but thinks rather that there is an alteration in what he terms the "physiological pattern" of the egg. In this same connection it can be noted that toxic agents applied to developing frog embryos can likewise produce a disproportion of parts most noticeably influencing those regions having the highest metabolic activity at the time of application (Bellamy, 1919).

With these results in mind, it is reasonable to suggest that the differences between the cytoplasm of the eggs of these two geographic forms or races are differences in factors which normally determine the varying rates of metabolism and cell division in the various parts of the developing blastula and possibly the induction processes in later stages. Only one bit of experimental evidence bearing on the physiological properties of these eggs is available and this of a very preliminary sort—the temperature tolerance is higher for the egg of the southern form. This, it is logical to suppose, is related to the fact that the southern embryos may be called upon to develop at higher temperatures than the northern. From this, however, it is not possible to reason that other physiological differences which may exist between the two eggs are likewise related to climatic influences.

It is realized that other subjects of interest could be discussed in relation to the results of these experiments but it is felt that they may be considered

more successfully after more information has been accumulated. For the present, it seems best to emphasize that the gametes of two geographic forms probably of the same species differ slightly in their cytoplasmic and nuclear properties and that by androgenetic haploid as well as diploid hybridization the orderly and measurable effects of these properties on early morphogenesis can be observed. The nature of these differences, their mode of operation, the relation of the embryonic differences they produce to the differences between the adults are among the major problems which can be and should be examined later with the same or similar materials and methods.

SUMMARY

1. Two distinct forms of frog, commonly referred to as *Rana pipiens*, Schreber, are described, and evidence is presented to show that they probably represent geographic races of that species, one from northern Vermont, the other from southeastern Pennsylvania.

2. In the experiments described, the gametes of these two races have been combined reciprocally to form diploid and androgenetic haploid hybrids and the early development of these has been studied in detail.

3. The diploid hybrids developed through metamorphosis; the androgenetic hybrids for 7 to 11 days, up to about stage 24 (Shumway, 1940).

4. A comparison of 3-day-old control and hybrid embryos reveals that, in general, the combinations which include cytoplasm of the northern form are characterized by larger head primordia and smaller posterior axial structures than are observed in those with southern cytoplasm. Such dissimilarities, only slightly apparent between the homospermic diploid controls, become progressively more accentuated between the homospermic haploids, the heterospermic (hybrid) diploids, and the heterospermic (hybrid) haploids.

5. A study of gastrula, neurula, and older stages discloses the early expressions and later fate of the dissimilarities shown by the 3-day-old embryos.

6. These results demonstrate:

(a) Cytoplasmic differences between the eggs of the two forms which seem to have contrasting effects upon the same developmental processes.

(b) Nuclear differences which, in homospermic diploid control development, appear to compensate for the cytoplasmic differences.

(c) An orderly cytoplasmic influence on early morphogenesis.

7. The possible nature and mode of action of these differences are briefly discussed.

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THE QUANTITY OF IRRADIATED NON-VIRULENT RABIES VIRUS REQUIRED TO IMMUNIZE MICE AND DOGS

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In a previous paper we pointed out that 1.5 cc. of irradiated tissue culture virus successfully immunizes mice against a subsequent intracerebral or intramuscular test infection (1). It soon developed, however, that extremely large volumes of the irradiated culture virus were needed to immunize dogs. Quantitative studies were undertaken, therefore, to determine the amounts of vaccine necessary for mice and dogs in terms of mouse intracerebral lethal doses.

Minimum Volume and Virus Content of Culture Virus Required for Immunization of Mice

The first experiments dealt with the minimum dose of tissue culture vaccine capable of immunizing mice. In the previous paper (1), we stated that the culture virus usually titred 0.03 cc. of the 10^{-2} dilution in 4 to 6 weeks old mice and was therefore said to contain 33,000 mouse intracerebral lethal doses per cc. 1.5 cc., or approximately 50,000 doses, properly irradiated, immunized the mice. More recently we have adopted as a standard for titration 3 weeks old mice rather than those aged 4 weeks or more. In these younger mice the culture virus often titres one dilution higher, 0.03 cc. of the 10^{-4} dilution.

Repeated experiments to determine the least amount of irradiated tissue culture virus¹ necessary to immunize mice indicated that 50,000 mouse doses, properly irradiated, gave good protection, whereas less than this amount, although sometimes effective, was not consistently so. The following experiments illustrate the type of result obtained.

Experiment 1.—Tissue culture virus, Pasteur strain,² prepared as previously described (1), was distributed in three quartz flasks. One was irradiated 20 minutes and then

¹ We are indebted to Dr. George I. Lavin for irradiating the preparations used in these experiments.

² This strain was kindly sent by Dr. Pierre L  pine of the Pasteur Institute, Paris, and has been passaged in mice in our laboratory.

tested for virulence, the second 30 minutes and tested, and the third 40 minutes and tested for virulence by inoculating 0.03 cc. intracerebrally into each of five mice. The 30 minute irradiated virus was then injected as a vaccine intraperitoneally into 30 day old Swiss mice in the following manner. Group 1 received 1 cc. in a single injection; group 2, an injection of 0.5 cc. and 2 days later a second injection of 0.5 cc., and group 3 received 0.2 cc. every other day for five injections. Group 4 received 0.5 cc. in a single injection; group 5, 0.25 cc., and 2 days later a second injection of 0.25 cc. Group 6 received 0.1 cc. daily for 3 days and finally, group 7 received no vaccine.

3 weeks after commencing vaccination the mice were tested for immunity by injecting them into the gastrocnemius muscle with 0.01 cc. of virulent rabies virus in serial two-fold dilutions, four mice being employed for each dilution. All mice were observed 4 weeks for signs of rabies.

The virulence of the virus before irradiation (Table I) proved to be 0.03 cc. of the 10^{-4} dilution or better. Hence the material is said to contain 330,000 mouse doses per cc. or more. Following irradiation for 20, 30, or 40 minutes, the material injected intracerebrally into mice failed to kill. In the immunity test, the non-vaccinated mice titred 1 to 1,800 according to the method of Reed and Muench (2), whereas the batches of vaccinated mice withstood 23.5 and 47+ times this amount, indicating a strong immunizing potency of the vaccine. In this experiment, 111,000 or more doses of irradiated vaccine given by any of the above methods immunized effectively.

Experiment 2.—Tissue culture virus was prepared, irradiated, and tested for virulence as in Experiment 1. 0.5 cc. of the 20 minute irradiated virus was injected as a vaccine intraperitoneally into a batch of 30 day old Swiss mice. The 30 and 40 minute preparations were likewise injected into a second and third batch. A fourth batch received three injections of 0.5 cc. of the 30 minute irradiated vaccine and the fifth batch was left unvaccinated as controls. 3 weeks after commencing vaccination the vaccinated and unvaccinated mice were given a test dose of virulent virus intramuscularly, as described in Experiment 1, and observed 4 weeks for signs of rabies.

The results of this test are shown in Table II. The virulence of the virus before irradiation proved to be 0.03 cc. of the 10^{-3} dilution. Hence the material is said to contain 33,000 mouse doses per cc. Following irradiation three of the five mice injected with the 20 minute material succumbed to rabies, two of the five injected with the 30 minute, and none injected with the 40 minute irradiated virus. Only this latter preparation was regarded, therefore, as non-virulent. In the immunity test the challenge virus in the non-vaccinated mice titred 1 to 1,280. The mice vaccinated with 16,500 doses withstood three times and those vaccinated with 49,500 doses withstood thirty-one times as much virus, indicating a strong immunity. In passing, it may be noted also that, even though most of the vaccine given in this experiment contained a trace of virulent virus, it was too small in quantity to affect the immunizing potency of the material (1).

TABLE I

*Immunization of Mice with Graded Doses of Irradiated Tissue Culture Pasteur Rabies Virus*Virulence before irradiation (0.03 cc. in dilutions intracerebrally) 10^{-2} - $3/4^*$: 10^{-3} - $2/4$: 10^{-4} - $2/4$.

" following " (0.03 cc. undiluted ") 20 minutes - 0/5:30 minutes - 0/5.

Immunity of Vaccinated Mice

| Dose of vaccine | Mortality of mice given test virus intramuscularly (0.01 cc.) in dilutions | | | | | | | Titret of virus in mice | Difference in titre of virus in vaccinated and non-vaccinated mice |
|----------------------------------------|----------------------------------------------------------------------------|-------|-------|-------|---------|---------|---------|-------------------------|--------------------------------------------------------------------|
| | 1/80 | 1/160 | 1/320 | 1/640 | 1/1,280 | 1/2,560 | 1/5,120 | | |
| Group 1: 1 cc., 1 dose (330,000 M.D.†) | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 | — | — | <80 | 47+ |
| " 2: 0.5 cc., 2 doses " | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 | — | — | <80 | 47+ |
| " 3: 0.2 cc., 5 " " | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 | — | — | <80 | 47+ |
| " 4: 0.5 cc., 1 dose (165,000 M.D.) | 2/4 | 0/4 | 0/4 | 0/4 | 0/4 | — | — | 80 | 23.5 |
| " 5: 0.25 cc., 2 doses " | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 | — | — | 80 | 23.5 |
| " 6: 0.1 cc., 3 " (111,000 ") | 0/4 | 1/4 | 0/4 | 0/4 | 0/4 | — | — | <80 | 47+ |
| " 7: No vaccine | — | 2/4 | 4/4 | 3/4 | 3/4 | 3/4 | 0/4 | 1,800 | |

* $3/4$ = 3 of 4 mice injected died of rabies. † Estimated by method of Reed and Muench (2).

‡ Mouse intracerebral lethal doses in vaccine prior to irradiation. — = material not tested.

TABLE II

*Immunization of Mice with Graded Doses of Irradiated Tissue Culture Pasteur Rabies Virus*Virulence before irradiation (0.03 cc. in dilutions intracerebrally) 10^{-2} - $3/3^*$: 10^{-3} - $3/3$: 10^{-4} - 0/3.

" following " (0.03 cc. undiluted ") 20 minutes - 3/5:30 minutes - 2/5:40 minutes - 0/5.

Immunity of Vaccinated Mice

| Dose of vaccine | Mortality of mice given test virus intramuscularly (0.01 cc.) in dilutions | | | | | | | Titret of virus in mice | Difference in titre of virus in vaccinated and non-vaccinated mice |
|------------------------------------------------------------|----------------------------------------------------------------------------|------|-------|-------|-------|---------|---------|-------------------------|--------------------------------------------------------------------|
| | 1/40 | 1/80 | 1/160 | 1/320 | 1/640 | 1/1,280 | 1/2,560 | | |
| Group 1: 0.5 cc., 1 dose (16,500 M.D.†). Irradiated 20 min | 4/4 | 2/4 | 3/4 | 2/4 | 1/4 | 2/4 | — | 320 | 4.0 |
| " 2: Same dose. Irradiated 30 min | 4/4 | 3/4 | 3/4 | 3/4 | 2/4 | 0/4 | — | 416 | 3.1 |
| " 3: Same dose. Irradiated 40 min | 3/3 | 3/4 | 2/4 | 3/4 | 2/4 | 1/4 | — | 415 | 3.1 |
| " 4: 0.5 cc., 3 doses (49,500 M.D.). Irradiated 30 min | 0/4 | 1/4 | 0/4 | 0/4 | 0/4 | 0/4 | — | <40 | 31.2 |
| " 5: No vaccine | — | — | 3/4 | 3/4 | 2/4 | 3/4 | 1/4 | 1,280 | |

Footnotes the same as in Table I.

TABLE III

*Immunization of Mice with Graded Doses of Concentrated Irradiated Tissue Culture Pasteur Rabies Virus*Virulence before irradiation (0.03 cc. in dilutions intracerebrally) 10^{-2} - $2/2^*$: 10^{-3} - $2/2$: 10^{-4} - $1/2$: 10^{-5} - 0/2.

" following " (0.03 cc. undiluted ") 45 minutes - 0/4.

Immunity of Vaccinated Mice

| Dose of vaccine | Mortality of mice given test virus intramuscularly (0.01 cc.) in dilutions | | | | | | Titret of virus in mice | Difference in titre of virus in vaccinated and non-vaccinated mice |
|--------------------------------------------------------------|----------------------------------------------------------------------------|-------|-------|-------|---------|--|-------------------------|--------------------------------------------------------------------|
| | 1/80 | 1/160 | 1/320 | 1/640 | 1/1,280 | | | |
| Group 1: 1.5 cc., 1 dose (49,500 M.D.†) | 0/4 | 0/4 | 0/4 | 0/4 | — | | <80 | 6.3+ |
| " 2: 0.15 cc., 1 dose (49,500 M.D.) concentrated 10 times | 0/4 | 0/4 | 0/4 | 0/4 | — | | <80 | 6.3+ |
| " 3: 0.5 cc., 1 dose (16,600 M.D.) | 3/4 | 2/4 | 1/4 | 0/4 | — | | 160 | 3.2 |
| " 4: 0.5 cc., 1 dose (166,000 M.D.) concentrated 10 times .. | — | 0/2 | 0/3 | 0/3 | — | | <160 | 3.2+ |
| " 5: 0.01 cc., 1 dose (3,300 M.D.) concentrated 10 times | 3/4 | 3/4 | 2/4 | 0/3 | — | | 320 | 1.6 |
| " 6: No vaccine | — | 3/4 | 3/4 | 1/4 | 0/3 | | 400 | |

Footnotes the same as in Table I.

Protocols with further data are shown (Table III), supporting the conclusion that approximately 50,000 irradiated doses of culture virus are required to immunize mice.

Minimum Volume and Virus Content of Culture Virus Required for Immunization of Dogs

The findings with mice led to a rough assay of the amount of vaccine required to immunize dogs. The following experiment shows that beagle dogs of the sort used in previous work with vaccines (3), weighing 10 kilos, or about 500 times as much as 20 gm. mice, were not immunized by 75 times but were immunized to some extent by 500 times the mouse dose of irradiated culture vaccine.

Experiment 3.—Tissue culture virus was prepared, tested, irradiated 40 minutes, and tested again for virulence as in the previous tests. Four beagle dogs, 4 to 6 months old and weighing 14 to 16 pounds, each received 450 cc. of the irradiated vaccine intraperitoneally and another batch of four similar dogs each received 75 cc. Five additional dogs were set aside as controls. 3 weeks later, each received 0.25 cc. of virulent virus diluted 1 to 200 (about one lethal dose (3)) into the neck muscles of the right and left sides. They were observed for signs of rabies for 2½ months.

The culture virus before irradiation titred 10^{-8} and hence contained 33,000 mouse doses per cc. Following irradiation the vaccine was not virulent for the mice. All five non-vaccinated dogs succumbed to the test virus on the 12th, 13th, 14th, 23rd, and 34th days and those receiving 75 cc. on the 15th, 16th, 19th, and 25th days respectively, whereas of the four receiving 450 cc., one died of rabies on the 14th and one on the 63rd days, and the other two remained well.

In view of the fact that dogs such as those used in our tests (3) needed as much as 500 cc. of irradiated vaccine as prepared to become immune to the test virus, and hence of a possible relation between weight of animal and amount of vaccine required, attempts were made to secure a virus preparation with a greater number of mouse doses per cc. Efforts to increase the titre of the culture virus have thus far been unsuccessful. Concentration procedures, on the other hand, were encouraging as far as they were carried out.

Experiment 4.—Tissue culture virus was centrifuged at 1,000 R.P.M. for 5 minutes, the supernatant drawn off, tested for virulence, irradiated 45 minutes with the mercury vapor lamp, and tested again for virulence. 116 cc. of this relatively clear material, free of obvious tissue fragments, were evaporated to dryness at low temperature in reduced atmospheric pressure and then resuspended in distilled water to 11.6 cc., or one-tenth of its original volume.

Sixteen mice were injected intraperitoneally with 1.5 cc. of the unconcentrated and sixteen mice with 0.15 cc. of the concentrated vaccine. Moreover, sixteen were injected with 0.5 cc. of the unconcentrated and sixteen with 0.5 cc. of the concentrated vaccine. Sixteen mice were given 0.01 cc. of the concentrated vaccine and sixteen mice remained unvaccinated. 3 weeks later, all mice were tested for immunity by injecting them intramuscularly with 0.01 cc. of virulent rabies virus in graded doses. They were observed for signs of rabies for 4 weeks.

The results of this experiment are shown in Table III. The titre of the virus before irradiation was 0.03 cc. of the 10^{-3} dilution. Following irradiation it failed to kill the inoculated mice. In the immunity test, the virus in unvaccinated mice titred 1 to 400. Mice dying from causes other than rabies are not included in the table. All mice receiving 49,500 mouse doses in a 1.5 cc. volume (group 1) and in the concentrated 0.15 cc. volume (group 2) remained well. Mice receiving 16,600 doses in 0.5 cc. (group 3) showed little immunity, whereas those receiving 166,000 doses in 0.5 cc. (group 4) remained well. Finally, the mice receiving only 3,300 doses were not immunized. 50,000 or more mouse doses, even in a ten times concentrated volume, remained capable of conferring a high grade immunity. Similar tests have not been performed on dogs.

Minimum Volume and Virus Content of Mouse Brain Tissue Virus Required for Immunization of Mice

Known sources of rabies virus other than tissue culture are limited largely to mammalian brain tissue. Brain tissue has the advantage of affording the largest yield of virus and consequently is the standard source of vaccines, yet it has the concomitant disadvantage of accompanying the virus in concentrations of from 4 to 33 per cent. Experiments were undertaken, therefore, to determine whether virus could be readily separated from brain tissue without loss of titre or immunizing potency.

Comparative titrations on supernatants and sediments of centrifuged mouse brain preparations indicated that speeds of 3,000 R.P.M. for 20 minutes removed a large part of the brain tissue without appreciable loss of virus.

Experiment 5.—The Pasteur strain of rabies virus was injected intracerebrally into the brains of four 3 weeks old W-Swiss mice. 6 days later, when they became prostrate, the brains of two were removed, ground in a mortar, and made up to a 10 per cent suspension in water. The material was then centrifuged 20 minutes at 3,000 R.P.M. in a Swedish angle centrifuge. The supernatant was removed and the sediment brought back to its original volume in serum water. Further dilutions of supernatant and sediment were made and titred intracerebrally in Swiss mice.

The results of the titrations are shown in Table IV. Both the supernatant and sediment titred as usual through the 10^{-7} dilution, indicating that as much titrable virus per cc. was contained in the relatively clear supernatant as in the thick brain-tissue-containing sediment.

Having learned that suspensions of mouse brain virus can be cleared by centrifugation without demonstrable loss of virulence, experiments were made to determine the relative immunizing potency of uncentrifuged and cleared vaccine.

Experiment 6.—9 cc. of 33 per cent commercial chloroformized antirabies canine vaccine were diluted with 81 cc. of horse serum water to make a 3.3 per cent suspension. 40 cc. of this suspension were used as a vaccine on one batch of mice. The remaining 50 cc. were centrifuged at about 2,500 R.P.M. for 15 minutes, after which the supernatant became lightly opalescent and largely free of gross particles. This supernatant was used as a vaccine on the second batch of mice.

TABLE IV

Comparative Titre of Rabies Virus in Supernatant and Sediment of Brain Tissue Suspensions Centrifuged at 3,000 R.P.M. for 20 Minutes

| Material | Fate of mice injected intracerebrally with virus suspensions (0.03 cc.) in dilutions | | | |
|------------------|--------------------------------------------------------------------------------------|-----------|-----------|-----------|
| | 10^{-4} | 10^{-5} | 10^{-6} | 10^{-7} |
| Supernatant..... | 4/4 | 4/4 | 4/4 | 3/4 |
| Sediment..... | 4/4 | 4/4 | 2/4 | 3/4 |

Each mouse received a total of 1 cc. of the given vaccine—one-half of each batch in a single dose and the other in five doses of 0.2 cc. each. 3 weeks later the four batches of vaccinated plus a fifth batch of unvaccinated mice were tested for their immunity to virulent virus injected in 0.01 cc. doses in twofold dilutions into the gastrocnemius muscle.

The results of the test (Table V) show that the test virus in unvaccinated mice titred 1 to 1,600. The mice given uncentrifuged material withstood at least twenty times and those given centrifuged material withstood five and twenty times this amount of virus respectively. Apparently the centrifuged supernatant confers an immunity of the same order as that of uncentrifuged material.

Further experiments showed that virus-containing supernatants prepared as above could be readily irradiated until virulence was destroyed and yet retain their immunizing potency.

Experiment 7.—Fifteen 3 weeks old W-Swiss mice were inoculated intracerebrally with 0.03 cc. of the rabies Pasteur strain diluted 1 to 100 in serum water. 6 days later the mice were prostrate, sacrificed, and their brains removed. The brains were emulsified

with diluent to make a 10 per cent suspension. This suspension was then titrated for virulence.

18 cc. were placed in a quartz flask, 9 cc. plus 9 cc. of diluent in a second flask, and finally, 1.5 cc. plus 13.5 cc. in a third flask to give final dilutions of 10 per cent, 5 per cent, and 1 per cent for irradiation. Irradiation was then commenced and samples were

TABLE V
*Immunization of Mice with Graded Doses of Centrifuged and Uncentrifuged Commercial
33 Per Cent Chloroformed Rabies Vaccine*
Immunity of Vaccinated Mice

| Dose of vaccine | Mortality of mice given test virus intramuscularly (0.01 cc.) in dilutions | | | | | | | Titre* of virus in mice | Difference in titre of virus in vaccinated and non- vaccinated mice |
|------------------------------------------------|-------------------------------------------------------------------------------|-------|-------|-------|---------|---------|---------|-------------------------------|---------------------------------------------------------------------------------------|
| | 1/80 | 1/160 | 1/320 | 1/640 | 1/1,280 | 1/2,560 | 1/5,120 | | |
| Group 1: 1 cc., 1 dose.... | 0/4† | 0/4 | 1/4 | 0/4 | 0/4 | — | — | <80 | 20+ |
| “ 2: 0.2 cc., 5 doses | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 | — | — | <80 | 20+ |
| “ 3: 1 cc., 1 dose (super- natant) | 3/4 | 0/4 | 1/4 | 1/4 | — | 1/4 | — | 320 | 5 |
| “ 4: 0.2 cc., 5 doses (super- natant). | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 | — | — | <80 | 20+ |
| “ 5: No vaccine ... | — | — | 3/4 | 3/4 | 3/4 | 0/4 | 2/4 | 1,600 | |

* Estimated by method of Reed and Muench (2).

† 0/4 = none of 4 mice injected died of rabies.

TABLE VI
Virulence of Mouse Brain Rabies Virus Following Irradiation with a Quartz Mercury Vapor Lamp
Virulence of 10 per cent emulsion before irradiation 10^{-5} - $4/4$: 10^{-6} - $4/4$: 10^{-7} - $0/4$:
 10^{-8} - $0/4$.

| Concentration <i>per cent</i> | Fate of mice injected with undiluted material irradiated: | | | | | | |
|----------------------------------|-----------------------------------------------------------|---------|---------|---------|---------|---------|---------|
| | 20 min. | 30 min. | 40 min. | 50 min. | 60 min. | 70 min. | 80 min. |
| 10 | 4/4 | 4/4 | 3/4 | 2/4 | 0/4 | 0/4 | 0/4 |
| 5 | 1/3 | 0/2 | 0/3 | 0/4 | 0/4 | 0/4 | 0/4 |
| 1 | 0/4 | 0/4 | 0/4 | — | — | — | — |

* $4/4$ = 4 of 4 mice injected died of rabies.

— = material not tested.

withdrawn for virulence tests at 20 minutes and at 10 minute intervals thereafter for a total of 80 minutes.

The results of the various virulence tests are shown in Table VI.

The virulence end point of the material before irradiation proved to be 0.03 cc. of the 10^{-6} dilution. The 10 per cent emulsion proved virulent after 50 minutes' but not longer irradiation; the 5 per cent after 20 but not

longer, and the 1 per cent not after 20 minutes' irradiation. Both the 5 per cent and the 1 per cent preparations appeared to be as readily inactivated by ultraviolet light as the tissue culture materials.

Experiment 8.—A 1 per cent suspension of rabies virus was prepared as above and tested for virulence. A portion was set aside for inactivation with chloroform (Experiment 10) and a portion irradiated, and samples were tested for virulence at 10, 20, and 30 minutes. The bulk of the vaccine after 30 minutes' irradiation was stored in the

TABLE VII

Immunization of Mice with Graded Doses of 1 Per Cent Irradiated and 1 Per Cent Chloroformized Mouse Brain Rabies Virus

Virulence before irradiation (0.03 cc. in dilutions intracerebrally) 10^{-5} - $4/4^*$: 10^{-6} - $4/4$:
 10^{-7} - $3/3$: 10^{-8} - $2/4$.
 " following " (0.03 cc. undiluted ") 10 minutes - $5/5$:20 minutes - $5/5$:30 minutes - $2/10$:30 plus 5 minutes - $0/5$.

Immunity of Vaccinated Mice

| Dose of vaccine | Mortality of mice given test virus intramuscularly (0.01 cc.) in dilutions | | | | | | | | Titre of virus in mice | Difference in titre of virus in vaccinated and non- vaccinated mice |
|------------------------------------------------------------|-------------------------------------------------------------------------------|------|------|------|-------|-------|-------|---------|------------------------------|---------------------------------------------------------------------------------|
| | 1/10 | 1/20 | 1/40 | 1/80 | 1/160 | 1/320 | 1/640 | 1/1,280 | | |
| <i>Experiment 8. Irradiated vaccine</i> | | | | | | | | | | |
| Group 1: 1 per cent 0.5 cc. (1,650,000 M.D.) irradiated | 1/5 | 2/5 | 0/5 | 0/5 | 0/5 | 1/5 | — | — | 10 | 80 |
| " 2: 1 per cent 0.1 cc (330,000 M.D.) irradiated | 2/5 | 0/5 | 1/5 | 0/5 | 1/5 | 0/5 | — | — | 11 | 72 |
| " 3: 0.1 per cent 0.1 cc. (33,000 M.D.) irradiated | 1/5 | 0/5 | 1/5 | 2/5 | 2/5 | 2/5 | — | — | 16 | 50 |
| " 4: No vaccine | — | — | 4/5 | 4/5 | 3/5 | 4/6 | 4/5 | 4/5 | 1/4 | 800 |
| <i>Experiment 11. Chloroformized vaccine</i> | | | | | | | | | | |
| Group 1: 1 per cent 0.1 cc (330,000 M.D.) | 2/4 | 2/4 | 0/4 | 3/5 | 1/5 | 1/5 | — | — | 31 | 26 |
| " 2: 0.1 per cent 0.1 cc (33,000 M.D.) | 4/5 | 3/5 | 2/5 | 2/5 | 2/5 | 1/5 | — | — | 48 | 16.5 |

Footnotes the same as in Table I.

ice box to await the outcome of the virulence test. The material before irradiation titred very high, 0.03 cc. 10^{-8} —and following 30 minutes' irradiation was still fatal to two of ten injected mice. Consequently it was given a second irradiation of 5 minutes, 8 days after the first. This time the preparation proved non-virulent.

The vaccine, then 13 days old, was given to mice intraperitoneally as follows: One group received 0.5 cc. of the 1 per cent suspension; a second group, 0.1 cc. of the 1 per cent, and a third group, 0.1 cc. of the 1 per cent diluted ten times to make a 0.1 per cent suspension. 3 weeks later these mice, together with controls, were tested for resistance to an intramuscular injection of virulent virus administered as described above.

The results are shown in Table VII. According to the high titre of the virus before irradiation, the mice given 0.5 cc. of the 1 per cent suspension

received at least 1,650,000 doses; those given 0.1 cc., 330,000 doses, and those given 0.1 cc. of the 0.1 per cent suspension, 33,000 doses. In the immunity test, challenge virus in the unvaccinated mice titred to an end point of 0.01 cc. of 1 to 800 dilution; the mice vaccinated with 1,650,000 doses withstood eighty times as much virus, those vaccinated with 330,000 doses, seventy-two times, and with 33,000 doses, fifty times as much virus. Apparently irradiated mouse brain virus gives clear-cut protection in doses of 33,000.

TABLE VIII

Immunisation of Mice with Graded Doses of 1 Per Cent Irradiated Mouse Brain Rabies Virus

Virulence before irradiation (0.03 cc. in dilutions intracerebrally) 10^{-5} - $4/4^*$: 10^{-8} - $4/4$:
 10^{-7} - $2/4$: 10^{-8} - $0/4$.

" following " (0.03 cc. undiluted ") 35 minutes - $0/6$.

Immunity of Vaccinated Mice

| Dose of vaccine | Mortality of mice given test virus intramuscularly (0.01 cc.) in dilutions | | | | | Titre† of virus in mice | Difference in titre of virus in vaccinated and non-vaccinated mice |
|-----------------------------------------|----------------------------------------------------------------------------|------|-------|-------|---------|-------------------------|--------------------------------------------------------------------|
| | 1/10 | 1/40 | 1/160 | 1/640 | 1/2,560 | | |
| No vaccine (A)..... | 5/5 | 5/6 | 5/6 | 1/6 | 2/6 | 368 | |
| " " (B)..... | 5/5 | 5/5 | 4/5 | 1/5 | 1/5 | 384 | |
| 1 per cent 0.2 cc. (660,000 M.D.†)..... | 1/6 | 0/6 | 1/6 | 0/6 | 0/6 | <10 | 37.5+ |
| 1 per cent 0.1 cc. (330,000 M.D.)..... | 3/5 | 3/5 | 0/5 | 1/5 | 0/5 | 40 | 9.6 |
| 0.1 per cent 0.1 cc. (33,000 M.D.)..... | 5/5 | 4/5 | 3/5 | 0/5 | 0/5 | 160 | 2.4 |
| No vaccine (C)..... | 5/7 | 4/5 | 2/5 | 0/5 | — | 40 | |
| 1 per cent 0.1 cc. (330,000 M.D.)..... | 1/7 | 0/6 | 1/6 | 0/6 | — | <10 | 4+ |

Footnotes the same as in Table I.

Further tests showed that the critical dose for immunizing these mice was roughly in the neighborhood of 50,000, an amount similar to that found necessary for tissue culture virus.

Experiment 9.—A 1 per cent suspension of rabies virus was prepared as above and tested for virulence. It was then irradiated 35 minutes and tested again for virulence.

15 days later the vaccine was given intraperitoneally to mice as follows: One batch received 0.2 cc. of the 1 per cent vaccine and a second batch (A) was left unvaccinated as controls. 5 days later, a second batch of mice was given 0.1 cc. of the vaccine, a third batch, 0.1 cc. of the 1 per cent vaccine diluted ten times to make a 0.1 per cent preparation, and a fourth batch (B) was left unvaccinated as controls. At the same time another batch was given 0.1 cc. of the 1 per cent vaccine and a final batch (C) set aside as controls.

3 weeks following vaccination, the mice were tested for immunity as follows. The first two series prepared on the 15th and 20th days were tested intramuscularly with a virulent passage strain and the final series with a street strain which had received no laboratory passages.

The results are shown in Table VIII. According to the virulence tests, the vaccinated mice received 660,000, 330,000, and 33,000 doses of irradiated virus respectively (Table VIII). The immunity test showed that the test virus in the non-vaccinated mice (A and B) titred to an end point of 0.01 cc. of the 1 to 368 and 384 dilutions respectively; the mice receiving 660,000 and 330,000 doses of vaccine withstood 37.5 and 9.6 times this amount respectively, indicating a considerable degree of immunity. Mice vaccinated with 33,000 doses withstood 2.4 times as much virus as non-vaccinated mice. Finally, street virus in non-vaccinated mice titred to the 1:40 dilution and mice vaccinated with 330,000 doses withstood at least four times as much virus, indicating a well marked immunity. In short, 33,000 doses barely immunized the mice, whereas more than this amount gave good protection.

Relative Immunizing Potency for Mice of Irradiated and Chloroformized Vaccines

The relative immunizing potencies of irradiated and chloroformized vaccines have been compared in five tests. Three of these showed no striking differences, whereas two showed a superiority of irradiated vaccines.

Experiment 10.—A 1 per cent suspension of mouse brain virus was divided into two parts; one was spun in a Swedish centrifuge at 3,000 R.P.M. for 30 minutes and the other in a horizontal centrifuge at 500 R.P.M. for 5 minutes. The supernatants were both removed, titrated for virulence, exposed to ultraviolet light for 20 minutes, and 18 days later 0.25 cc. was injected as a vaccine into mice every other day until four doses had been given. 3 weeks later the vaccinated plus the control mice were tested for immunity to an intracerebral injection of virulent street virus.

Both the Swedish and horizontal centrifuge supernatants titred 0.03 cc. of 10^{-7} dilution and were non-virulent after 20 minutes' irradiation. The results of the immunity test are shown in Table IX.

This experiment shows that 1 per cent brain virus supernatants, following centrifugation at 500 or 3,000 R.P.M., remain equally virulent; that they may be exposed to ultraviolet light, and rendered avirulent in 20 minutes; and finally that they immunize mice in 1 cc. doses against at least 10,000 intracerebral lethal doses of street virus.

Experiment 11.—This experiment was run in conjunction with Experiment 8. The portion of 1 per cent virus set aside for treatment with chloroform received chloroform to make a 1 per cent concentration. The suspension was then shaken for 2 minutes in a mechanical shaker, and for 2 minutes daily thereafter. The material proved virulent after 6 days when injected intracerebrally into mice but not after 11 days.

Batches of mice were injected with 0.1 cc. and 0.1 cc. of a 0.1 per cent suspension of

TABLE IX

Immunization of Mice with Graded Doses of 1 Per Cent Irradiated Mouse Brain Rabies Virus against Intracerebral Test Infection

Supernatant—horizontal centrifuge

Virulence before irradiation (0.03 cc. in dilutions intracerebrally) 10^{-5} - $4/4$: 10^{-6} - $4/4$:
 10^{-7} - $3/4$: 10^{-8} - $0/4$.

“ following “ (0.03 cc. undiluted “) 20 minutes - $0/5$.

Supernatant—Swedish centrifuge

Virulence before irradiation (0.03 cc. in dilutions intracerebrally) 10^{-5} - $4/4$: 10^{-6} - $3/4$:
 10^{-7} - $2/3$: 10^{-8} - $0/4$.

“ following “ (0.03 cc. undiluted “) 20 minutes - $0/5$.

Immunity of Vaccinated Mice

| Dose of vaccine | Mortality of mice given test virus intracerebrally (0.03 cc.) in dilutions | | | | | Titre† of virus in mice | Difference in titre of virus in vaccinated and non-vaccinated mice |
|------------------------------------------------|----------------------------------------------------------------------------|------------------|------------------|------------------|------------------|-------------------------|--------------------------------------------------------------------|
| | 10 ⁻¹ | 10 ⁻² | 10 ⁻³ | 10 ⁻⁴ | 10 ⁻⁵ | | |
| No vaccine..... | — | 3/3 | 4/4 | 1/4 | 3/4 | 10^{-5} + | |
| 1 per cent 0.25 cc., 4 doses (3,300,000 M.D.†) | | | | | | | |
| “Horizontal” supernatant..... | 0/4 | 0/4 | 0/4 | — | — | $<10^{-1}$ | 10,000+ |
| “Swedish” “..... | 0/4 | 0/4 | 0/4 | 0/2 | — | $<10^{-2}$ | 10,000+ |

Footnotes the same as in Table I.

TABLE X

Immunization of Mice with Graded Doses of Irradiated and Chloroformized Mouse Brain Rabies Virus

Virulence before irradiation (0.03 cc., 10^{-7} dilution intracerebrally).

“ following “ (0.03 cc. undiluted “) 35 minutes— $0/6$.

Immunity of Vaccinated Mice

| Dose of vaccine | Mortality of mice given test virus intramuscularly (0.01 cc.) in dilutions | | | | | | | Titre† of virus in mice | Difference in titre of virus in vaccinated and non-vaccinated mice |
|-----------------------------------------------------|----------------------------------------------------------------------------|------|------|-------|-------|-------|---------|-------------------------|--------------------------------------------------------------------|
| | 1/10 | 1/40 | 1/80 | 1/160 | 1/320 | 1/640 | 1/1,280 | | |
| Group 1: No vaccine. | — | — | 4/6 | 4/6 | 2/6 | 1/6 | 2/6 | 250 | |
| “ 2: 0.5 cc., irradiated (1,650,000 M.D.†) . . | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | — | <10 | 25+ |
| “ 3: 0.1 cc., irradiated (330,000 M.D.) . . . | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | — | <10 | 25+ |
| “ 4: 0.5 cc., chloroformized (1,650,000 M.D.) . . . | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | — | <10 | 25+ |
| “ 5: 0.1 cc., chloroformized (330,000 M.D.) | 1/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | — | <10 | 25+ |

Footnotes the same as in Table I.

the vaccine respectively. 3 weeks later these mice, together with those given the irradiated virus and the controls, received the test virus intramuscularly in twofold dilution.

The results of the test are shown in Table VII. The mice given chloroformized vaccine withstood 26 and 16.5 times as much virus as non-vaccinated mice but only one-third as much as the mice given equivalent doses of irradiated vaccine respectively.

Experiment 12.—A 1 per cent mouse brain virus was prepared as in Experiments 7 and 9. One portion was irradiated 35 minutes and proved non-virulent by mouse inoculation; a second portion was treated with 1 per cent chloroform and proved non-virulent after 15 days. 48 days later, batches of mice received a single intraperitoneal injection of 0.5 or 0.1 cc. of the irradiated or chloroformized vaccine respectively. 6 weeks later, all mice, together with unvaccinated controls, were given a test intramuscular injection of virulent virus in a dose of 0.01 cc. in graded twofold dilutions.

The results are shown in Table X. Test virus in the non-vaccinated mice showed a titration end point of 0.01 cc. of 1 to 250 dilution, as contrasted with less than 1 to 10 in all vaccinated mice. The twenty-fivefold difference indicates considerable immunity in both irradiated and chloroformized vaccine groups.

Relative Immunizing Potency for Mice of Irradiated Mouse and Dog Brain Vaccines

The next step in developing a vaccine was to compare the titres of virus in infected brains of young mice with those in young guinea pigs, rabbits, and dogs. Repeated tests showed that dogs alone, injected with virus intracerebrally when 1 month old, yielded brain tissue with virus titres equal to those in infected mouse brains, namely, 0.03 cc. of the 10^{-6} or 10^{-7} dilution. Vaccines were prepared with infected dog brains and results obtained which paralleled those with mouse brains.

Experiment 13.—An infected dog brain weighing 64 gm. was homogenized in a mechanical shaker, diluted with buffer to form a 1 per cent emulsion, titrated for virulence, spun in a horizontal centrifuge at 500 R.P.M. for 5 minutes, distributed in 35 cc. quantities in quartz flasks, irradiated 20 minutes with ultraviolet light, tested again for virulence, and stored in the ice box. 3 weeks later, groups of mice were vaccinated as follows: Group 1 received 0.2 cc. intraperitoneally and group 2 received 0.2 cc. of the 1 per cent vaccine diluted ten times. A third group received 0.2 cc. of a commercial chloroformized vaccine, a fourth 0.2 cc. of the same vaccine diluted ten times, and a final group was left unvaccinated as controls. 3 weeks later the immunity of the mice was tested by giving them 0.01 cc. of street virus intramuscularly in graded doses.

The 1 per cent centrifuged supernatant titred 0.03 cc. of the 10^{-7} dilution and, following irradiation, failed to kill mice. Results of the immunity test are shown in Table XI. The titre of street virus in the unvaccinated was approximately 0.01 cc. of the 1 to 160 dilution, whereas that in mice

TABLE XI

*Immunisation of Mice with Graded Doses of Irradiated and Chloroformised Dog Brain Rabies Virus*Virulence before irradiation (0.03 cc., 10^{-7} dilution intracerebrally).

" following " (0.03 cc. undiluted ") 20 minutes—0/6.

Immunity of Vaccinated Mice

| Dose of vaccine | Mortality of mice given test virus intramuscularly (0.01 cc.) in dilutions | | | Titre† of virus in mice | Difference in titre of virus in vaccinated and non-vaccinated mice |
|-----------------------------------------------|----------------------------------------------------------------------------|------|-------|-------------------------|--------------------------------------------------------------------|
| | 1/10 | 1/40 | 1/160 | | |
| Group 1: 1 per cent, 0.2 cc. irradiated. | 1/9 | 0/8 | 0/8 | <10 | 16+ |
| " 2: 0.1 per cent, 0.2 cc. irradiated..... | 5/9 | 4/8 | 1/8 | 40 | 4 |
| " 3: 20 per cent, 0.2 cc. chloroformized..... | 2/8 | 0/8 | 0/8 | <10 | 16+ |
| " 4: 2 per cent, 0.2 cc. chloroformized..... | 2/8 | 2/8 | 1/8 | 10 | 16 |
| " 5: No vaccine..... | 7/9 | 5/8 | 3/8 | <160 | |

Footnotes the same as in Table I.

TABLE XII

*Immunization of Mice with Graded Doses of Irradiated Dog Brain Rabies Virus**after 9 Months' Storage*Virulence before irradiation (0.03 cc., 10^{-7} dilution intracerebrally).

" following " (0.03 cc. undiluted intracerebrally) 30 minutes—0/6.

Immunity of Vaccinated Mice

| Dose of vaccine | Mortality of mice given test virus intracerebrally (0.03 cc.) in dilutions | | | | | | | Titre† of virus in mice | Difference in titre of virus in vaccinated and non-vaccinated mice |
|---------------------------------------------------------------|----------------------------------------------------------------------------|-----------|-----------|-----------|-----------|-----------|-----------|-------------------------|--------------------------------------------------------------------|
| | 10^{-2} | 10^{-3} | 10^{-4} | 10^{-5} | 10^{-6} | 10^{-7} | 10^{-8} | | |
| Group 1: No vaccine..... | — | — | 4/4 | 4/4 | 4/4 | 1/4 | 0/4 | 10^{-6} | |
| " 2: 1 per cent, 0.25 cc., 4 doses, irradiated 15–30 min..... | — | 1/4 | 1/4 | 1/4 | 0/4 | — | — | 10^{-4} | 100 |
| " 3: 1 per cent, same dose, irradiated 90–120 min..... | 0/2 | 1/4 | 0/4 | 0/4 | 0/4 | — | — | < 10^{-2} | 10,000+ |
| " 4: 6.6 per cent, same dose, chloroformized..... | — | 2/3 | 2/4 | 3/4 | 1/4 | — | — | 10^{-5} | 10 |
| | Mortality of mice given test virus intramuscularly (0.01 cc.) in dilutions | | | | | | | | |
| | 1/10 | 1/40 | 1/160 | 1/640 | 1/2,560 | | | | |
| Group 5: No vaccine..... | 3/3 | 4/4 | 1/4 | 1/4 | 2/4 | | | 640 | |
| " 6: 1 per cent, 0.2 cc., irradiated 15–30 min..... | 0/4 | 1/4 | 0/4 | 0/4 | 0/4 | | | <10 | 64+ |
| " 7: 1 per cent, same dose, irradiated 90–120 min..... | 2/4 | 0/4 | 0/4 | 0/4 | 0/4 | | | 10 | 64 |
| " 8: 6.6 per cent, same dose, chloroformized..... | 3/4 | 1/4 | 1/4 | 0/4 | 1/4 | | | 35 | 18 |

Footnotes the same as in Table I.

receiving the 1 per cent irradiated or 20 per cent chloroformized vaccines was at least sixteen times, and in mice receiving 0.1 per cent irradiated or 2 per cent chloroformized vaccine, four to sixteen times greater, demonstrating considerable immunizing potency of both the 1 per cent irradiated dog brain and the 20 per cent chloroformized preparations.

A final experiment is submitted to show the effectiveness of the 1 per cent irradiated dog brain virus after 9 months at ice box temperature.

Experiment 14.—A 1 per cent dog brain virus was prepared and tested for virulence according to the method described in the previous experiment. Portions were exposed to ultraviolet light for 15 and 30 minutes, and for 90 and 120 minutes. Samples were tested for virulence at varying intervals from 3 to 30 minutes. The original material titred 0.03 cc. $\times 10^{-7}$ and at 30 minutes virus failed to kill mice. The various samples were then stored in the ice box at 40°F.

9 months later, mice were treated as follows: Group 1 remained unvaccinated. Other groups of mice were vaccinated intraperitoneally in the following manner. Group 2 was given 0.25 cc. of the pooled 15 and 30 minute vaccines every other day four times; group 3 received the same dose of pooled 90 and 120 minute vaccines; group 4 was given the same dose of a commercial chloroformized vaccine diluted five times; group 5 was left as a second batch of controls; group 6 was given a single dose of 0.2 cc. of the 15 minute irradiated vaccine, group 7 a single dose of 0.2 cc. of the 120 minute irradiated vaccine, and group 8 the same dose of the chloroformized vaccine diluted 1 to 5. 3 weeks later the first four groups were tested for immunity to an intracerebral injection and groups 5 to 8 were tested for immunity to an intramuscular injection of virulent virus.

Table XII shows that a total of 1 cc. of 1 per cent irradiated dog brain vaccine immunized mice against 100 to 10,000 intracerebral lethal doses and at least 64 intramuscular doses. The chloroformized vaccine immunized mice against 10 intracerebral test doses and 18 intramuscular doses.

DISCUSSION

From a practical viewpoint, culture virus has not yet proved a satisfactory source of rabies vaccine, due chiefly to its low content of virus. To immunize, approximately 1 cc. is required for mice and 500 cc. for dogs,—about 5 per cent of the body weight.

The supernatant of centrifuged brain tissue virus, however, has proved a good source of vaccine. The virus content of infected brain tissue per unit volume is 1,000 times that of tissue culture. A 10 per cent emulsion can be centrifuged to sediment a large portion of the tissue fragments without lowering the titre of virus. The supernatant from a 1 to 5 per cent emulsion can be irradiated so as to destroy virulence without loss of immunizing potency. 0.1 cc. of such a preparation immunizes mice adequately,—0.5 per cent of the body weight.

Altogether the results of these experiments to date suggest that basically the immunizing potency of a vaccine is dependent upon virus content, that is, that the immunizing antigen is the virus particle. They indicate also that one intracerebral lethal mouse dose of a given strain of virus from tissue culture is equivalent in immunizing potency to one dose of the same strain from infected mammalian brain. Finally, the findings point to a relation between number of mouse lethal doses required to immunize and body weight.

The 1 per cent irradiated dog brain virus has proved an effective and practical vaccine for immunizing mice, and equal or superior to chloroformized vaccine. It is now being tested in dogs with promising results (4).

SUMMARY

In the experiments described above, we found with respect to tissue culture rabies virus that 1 cc., which contains approximately 50,000 mouse intracerebral lethal doses, properly irradiated, was required to immunize a mouse; 500 cc., which contain 25,000,000 doses, were required to immunize a 20 pound beagle dog.

Tissue culture virus concentrated ten times proved capable of immunizing mice in a dose one-tenth as large as that required for unconcentrated culture virus.

Brain virus suspensions were centrifuged so as to remove a large part of the tissue particles without striking loss in the virulence of the supernatant. The centrifuged supernatants of 1 to 5 per cent brain virus suspensions were irradiated so as to destroy virulence and yet retain immunizing potency.

Irradiated supernatants of mouse brain virus proved capable of immunizing mice as well as or better than similar supernatants treated with chloroform.

0.1 cc. of a 1 per cent irradiated dog brain virus containing approximately 50,000 mouse intracerebral lethal doses immunized mice effectively.

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HISTOPATHOLOGY OF CNS OF MICE INFECTED WITH VIRUS OF THEILER'S DISEASE (SPONTANEOUS ENCEPHALOMYELITIS)*

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Since 1937, when Theiler¹ first described spontaneous encephalomyelitis of albino mice (Theiler's disease; "mouse poliomyelitis") and the virus causing it, two major developments have given this disease renewed importance because they confirm the impression that one is dealing here with an infection more closely related to human poliomyelitis than is any other known disease. In the first place, the finding by Olitsky² and by Theiler and Gard³ of Theiler's virus in the intestinal contents and feces not only of mice showing the signs of the spontaneous or experimental malady but also of normal, young adult or mature animals, recalls similar results obtained in studies on human poliomyelitis.^{4, 5} Again another epidemiological feature is shown commonly by the two diseases, *i. e.*, the analogous incidence of paralytic cases of one in more than 5000.

The second recent development under consideration concerns the successful transmission by Armstrong⁶ of one strain (Lansing) of human poliomyelitic virus to the white mouse. This notable achievement enables one now to carry the comparison of the 2 diseases further than it was heretofore possible. The clinical resemblance of human and experimental (monkey) poliomyelitis on one side and mouse-encephalomyelitis on the other has been fully stressed before.¹⁻³ In the mouse, the two maladies are clinically indistinguishable.

In view of the striking similarities noted in many properties of the two diseases, it was thought desirable to study in detail the histopathology of spontaneous and experimental Theiler's disease (a) in the hope that clues

* The writers wish to thank Dr. M. Theiler for his coöperation and for Lansing-strain infected mice, and Mr. P. Haselbauer for his technical assistance.

¹ Theiler, M., *J. Exp. Med.*, 1937, **65**, 705.

² Olitsky, P. K., *Proc. Soc. Exp. Biol. and Med.*, 1939, **41**, 434; *J. Exp. Med.*, 1940, **72**, 113.

³ Theiler, M., and Gard, S., *J. Exp. Med.*, 1940, **72**, 49, 79.

⁴ For literature see Trask, J. D., and Paul, J. R., *Am. J. Public Health*, 1941, **31**, 239.

⁵ Sabin, A. B., and Ward, R., *J. Bact.*, 1941, **41**, 49.

⁶ Armstrong, C., *Public Health Rep.*, 1939, **54**, 1719.

might be yielded which in turn could be applied to problems in the pathogenesis of human poliomyelitis and (b) for the purpose, particularly, of comparing in the same host the pathology of the Lansing strain of poliomyelitis and of Theiler's disease.

Methods and Materials. The CNS of more than 30 mice sacrificed either at the height of spontaneous Theiler's disease, or at various stages of the experimental infection following intracerebral (*regio parietalis*), intranasal, intralingual, and intraabdominal inoculation of the virus deriving from intestinal contents of mice were studied. Virus introduced by peripheral routes induced paralysis in approximately 10 to 15% of inoculated 14-day-old mice. In most instances the entire head from which the skin and lower jaw had been removed, and the entire spinal column were fixed in Zenker's solution plus 10% glacial acetic acid (for decalcification) for 30 hours before washing. The skull and contents were cut semiserially, each sagittal section being 6 microns thick; the first 6 sections of each ribbon of 30 were retained for mounting. The spinal column was cut only at the middle of the cervical, thoracic, and lumbar levels. Thus about 150 sections of the CNS were cut from each animal, and more than 5000 in all were studied. Eosin-methylene-blue or hematoxylin-eosin stains were used.

This method was first employed by Sabin and Olitsky⁷ who showed thereby the definite and selective pathways taken in the mouse, from the site of inoculation through the CNS, by another neurotropic virus, vesicular stomatitis. The progression of poliomyelitic virus through the CNS of man and monkey has been delineated by Sabin,⁸ Bodian and Howe,⁹ and others.^{8b, 9}

Results of Microscopical Examination. We have not been able, however, to find such clear-cut patterns in the brain of mice having Theiler's disease. Instead, no significant differences in the types of lesion and their distribution were noted, regardless of the route of inoculation. Of particular interest is the fact that the olfactory bulbs exhibited no characteristic changes—either in those 8 mice receiving the virus intranasally and responding with paralysis, or in 22 others which were infected by other routes, or in the spontaneous disease. This is in agreement with what is found to be true in human poliomyelitis but is in sharp contrast to the bulb-lesions that characterize poliomyelitis in the monkey following intranasal instillation of virus.^{8a} In this relation it should be pointed out that human poliomyelitis and murine encephalomyelitis are natural diseases of man and mouse but monkey-poliomyelitis is an experimental infection, artificially induced. Other pathways that may, pos-

⁷ Sabin, A. B., and Olitsky, P. K., *J. Exp. Med.*, 1938, **67**, 201.

⁸ a. Sabin, A. B., and Olitsky, P. K., *J. Am. Med. Assn.*, 1937, **106**, 21; b. Sabin, A. B., *Am. J. Dis. Child.*, 1940, **60**, 1313.

⁹ Bodian, D., and Howe, H. A., *Brain*, 1940, **63**, 135.

sibly, be taken by intranasally instilled Theiler's virus are via the V nerve or the sphenopalatine (parasympathetic) or superior cervical (sympathetic) ganglia. It was noted, however, that the gasserian and superior cervical ganglia were affected not only after intranasal but also after intralingual and intracerebral inoculation of virus. In no instance have we been able to find lesions in the sphenopalatine ganglion.

Intracerebral injection of intestinal Theiler's virus in sufficient dosage was invariably followed by clinically apparent infection with an incubationary period extending from 7 to 37 (usually 10 to 20) days.² Animals so inoculated were sacrificed after 7 days, before clinical signs of infection (paralysis) were seen. In the brains, the most marked lesions were observed at the site of injection. The area immediately surrounding it showed endothelial swelling and proliferation chiefly of the smaller vessels and capillaries, and perivascular microglial and lymphocytic infiltration. Perivascular neuronal degeneration and, less often, necrosis also were present. The lesions extended from the site, rostrally to the septal area and caudally to the pontine level. They spread out mainly periventricularly.

In still later stages of the period of incubation after intracerebral inoculation (9th–11th day), the lesions had expanded over the adjacent structures but chiefly periventricularly. The vascular changes as mentioned were more in evidence, as was perivascular gliosis, the gliosis also occurring elsewhere, diffusely or focally. Neuronal degeneration and necrosis were more marked but neuronophagia was met with only infrequently and the cells active in this process were neuroglial rather than polymorphonuclear. The pathological changes rostrally, at the septum or *corpus striatum* were not extensive and were predominantly of mesodermal-glial type; caudally, from the level of the thalamus to that of the *substantia nigra* or pons-medulla, they were more prominently neuronal (degeneration, necrosis, neuronophagia; loss of neurons with resulting vacuolization of the stroma), while the mesodermal-glial reactions became less conspicuous (Fig. 1).

Thus, at this stage, when clinical signs were still absent and the cord was still free from detectable changes, the characteristic picture of the fully developed disease was noted in the brain, except that in the latter stage the degree of reactions was more pronounced. The areas then most constantly affected were the *substantia nigra*, *tegmentum*, reticular formation, olivary nuclei, nuclei of V and VIII nerves, red and dentate nuclei. Cortical lesions were few in number, mainly vascular in type and sometimes consisted of neuroglial infiltration.

After onset of paralysis, in addition to the cerebral lesions, the anterior horns of the spinal cord were regularly involved: They revealed the vascular and perivascular reactions, neuronal degeneration, necrosis, and neuronophagia. The neuronal changes, however, were never as extensive as they are

ordinarily in the monkey cords in experimental poliomyelitis—there was always a number of apparently normal neurons present and only a few polymorphonuclear cells were, as a rule, seen. It is of interest that certain neurons showed,



FIG. 1. *Substantia nigra* in encephalomyelitis of the mouse. To be noted are the endothelial (A) and perivascular (B) reaction and neuronal degeneration (C), necrosis (D), neuronophagia (E) and vacuolization of the stroma. $\times 500$.

usually at the onset of paralysis and chiefly in the anterior horns of the cord, the presence of Cowdry Type B, intranuclear inclusion-bodies, similar to those seen in human and monkey poliomyelitis.

The progression of pathological changes during the period of incubation following peripheral inoculation of Theiler's virus could not be studied in the same way, in view of the irregularity of production of clinically apparent infection by these means.

After peripheral inoculation when paralysis developed, however, the pathological picture differed in no significant manner from that seen after intracerebral injection (except for the more pronounced periventricular distribution in the latter), and was also similar to that shown by mice spontaneously attacked.

In conclusion, in the fully developed clinical disease the types of lesions and their distribution in the CNS were not essentially different when the malady was induced by various routes of inoculation, regardless of the length of incubationary period. As a rule, the rostral part of the brain showed mesodermoglia reactions predominantly, while the caudal, including the cord, exhibited more marked neuronal degeneration and destruction.

The type of lesions and their distribution in Theiler's disease in albino mice closely resembled the picture in this animal at corresponding periods after infection with the Lansing strain of human poliomyelitis.¹⁰

¹⁰ Lillie, R. D., and Armstrong, C., *Public Health Rep.*, 1940, **55**, 718.

VIRUS OF SPONTANEOUS ENCEPHALOMYELITIS FOUND IN INTESTINES OF NORMAL WILD GRAY MICE

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Practically all of more than 300 normal, adult, albino mice (Rockefeller Institute, Swiss, and other strains) thus far tested, harbored in their intestines the virus of spontaneous encephalomyelitis (Theiler's disease; "mouse poliomyelitis").^{1, 2} The experimental and the spontaneous forms of this disease have been shown to be closely similar to human and experimental (monkey) poliomyelitis; moreover, there is a resemblance in many properties of the viruses causing the respective maladies.^{1, 2} A recent study³ of albino mice revealed no essential difference between the lesions of experimental and spontaneous Theiler's disease and those⁴ of the experimental infection induced by the Lansing strain⁵ of human poliomyelitis.

Albino mice have occurred among gray mice under domestication and have then been inbred. They are used chiefly as stock animals in biological laboratories, while the animal from which they are derived, the wild gray mouse, is not only a commensal of man but in its predatory existence it can contaminate man's food with its excreta. The almost universal presence of a virus, having so much in common with that of human poliomyelitis, in the intestines and feces^{1, 2} of the albino mice, is therefore of less significance to an epidemiologist than would be the finding of Theiler's virus in the intestines of wild gray mice. Hence, a search was undertaken for Theiler's virus in the intestines of apparently normal, wild gray house-mice, and those most readily at hand were the ones trapped in one of the animal houses of the Rockefeller Institute.

At the outset it should be noted that these gray mice are as susceptible to Theiler's virus, given intracerebrally, as are their albino derivatives. The encephalomyelitis so induced in both strains is indistinguishable on clinical, pathological, and immunological grounds; furthermore, the experimental disease in gray mice is transmissible and can be re-passaged to albino mice.

¹ Olitsky, P. K., Proc. Soc. Exp. Biol. and Med., 1939, **41**, 434; *J. Exp. Med.*, 1940, **72**, 113.

² Theiler, M., and Gard, S., *J. Exp. Med.*, 1940, **72**, 79.

³ Olitsky, P. K., and Schlesinger, R. W., Proc. Soc. Exp. Biol. and Med., 1941, **47**, 79.

⁴ Lillie, R. D., and Armstrong, C., *Pub. Health Rep.*, 1940, **55**, 718.

⁵ Armstrong, C., *Pub. Health Rep.*, 1939, **54**, 1719.

The methods used have already been described.¹ Table I shows the results obtained in 6 experiments in each of which the intestinal material and, in parallel, the brains of from 2 to 5 gray mice were pooled.

The outcome of 5 of the 6 experiments clearly points to the presence of the encephalomyelitic virus in the intestines of the apparently normal, gray mice trapped at the Institute. Further studies revealed that the intestinal virus

TABLE I
Theiler's Virus in Intestines of Adult Wild Gray Mice

| Exp. No. | No. of mice used | Pooled materials used | Paralysis produced | Remarks |
|----------|------------------|-----------------------------------------------------------------------------|--------------------|-------------------------------------------------------------------------------------------------------------------------------------|
| 1 | 3 | 12% suspension entire intestinal tract 10% susp. of brain | 0/8* 0/8 | |
| 2 | 2 | 10% susp. of contents only of entire intestinal tract 10% susp. of brain | 7/8 0/7 | Brain to brain 2nd passage with 10^{-1} to 10^{-6} suspensions, 84/95. Similarly, 3rd passage with 10^{-1} suspension, 10/10. |
| 3 | 3 | 10% intest. contents 10% susp. of brain | 7/10 0/10 | |
| 4 | 5 | 10% intest. contents 10% susp. of brain | 4/14 0/6 | |
| 5 | 5 | 10% intest. contents 10% susp. of brain | 1/14 0/6 | |
| 6 | 4 | 10% intest. contents 10% susp. of brain | 2/14 0/6 | |

* The numerator represents the number showing paralysis and the denominator the number of albino mice injected.

deriving from the gray mice is not different from that obtained from albino strains in clinical, pathological, and immunological (cross-immunity) properties and also in its non-pathogenicity in *rhesus* monkeys.⁶ As in albino mice, no virus was detected in the brains of the normal gray mice; in the former, virus was found in the brain only when they exhibited clinically apparent Theiler's disease.^{1, 2}

Discussion. Based on lesser proportion of positive results of pools, it would

⁶ Olitsky, P. K., Proc. Soc. Exp. Biol. and Med., 1940, **45**, 339.

appear that not as many apparently normal gray mice harbor Theiler's virus in their intestines as do albino ones.^{1, 2} (Cf. Theiler⁷.)

Although rigid quarantine and isolation are practiced in the animal house, no such control can be applied to predatory, wild gray mice. Hence the possibility arises that they were contaminated with Theiler's virus in an endemic focus populated by virus-carrying albino mice. This has not as yet been proved a fact; meanwhile, 5 experiments in which were examined 7 house and 3 field gray mice, all trapped at a distance from the laboratory, failed to show virus in their intestines or brains. Dr. Max Theiler reports, in addition, that virus was not detected in a much larger series of gray mice trapped away from the Institute.⁷

Conclusions. (1) Wild gray house-mice are as susceptible to intracerebral introduction of Theiler's virus as are albino mice. (2) Apparently normal gray mice trapped in an animal house in which almost all adult albino mice are carriers of Theiler's virus, reveal this virus in their intestines and not in their brains. (3) The virus is indistinguishable from that obtained from the intestines of normal albino mice. (4) The number of gray mice shown to be carriers of Theiler's virus, while considerable, appears to be lower than that of carriers found among the albino strain.

Preliminary tests to the present time have failed to indicate that the virus is demonstrable in gray house and field mice trapped at a distance from the laboratory. This subject is still under investigation.

⁷ Theiler, M., Studies on Poliomyelitis, De Lamar Lecture, in press.

THE TRANSMISSION OF AN INDUCED LYMPHATIC LEUKEMIA AND LYMPHOSARCOMA IN THE RAT*

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Evidence accumulated during recent years has made it progressively more evident that the essential factors in neoplasia and leukemia vary only in the type of cell involved in the malignant process. The attempts to produce leukemia experimentally by chemical carcinogenic agents have succeeded with some frequency in mice (3, 4, 10). As the spontaneous disease is not uncommon in these animals, Richter and MacDowell (13) have cautioned against overlooking the influence of genetic variables in the claims for the successful inductions. Certainly in some of the strains used for these studies, leukemia is known to occur spontaneously, as pointed out by Mider and Morton (9). However, these latter investigators leave little doubt that the carcinogenic chemicals at least bring out the disease at an earlier age and in a higher percentage of individuals than would occur spontaneously. It would thus appear that induced leukemia is probably another example of the part played by genetic constitution on the type of response which may take place to carcinogenic agents, a fact first demonstrated in this laboratory by Lynch (7, 8).

The present report has to do with the induction and transmission of leukemia in rats. This appears to be a rare condition, for among 489 tumors observed by Bullock and Curtis (2) in autopsies on over 2,400 rats, there were a number of thymus and lymph node tumors but no definite instance of leukemia. In a recent report (12) on the large Wistar Institute rat colony, only 2 out of 273 tumors which have occurred in this strain were of the lymphoid system and no cases of leukemia were mentioned. As the Wistar strain was used in the present study, the absence of leukemia and very low instance of lymphoid tumors is of particular interest. Ito (6) reports a single instance of myelogenous leukemia in the rat which he considers to have been induced by feeding amidoazo-toluol.

Method.—In the course of a study of the effect of different solvents on the carcinogenic property of 1,2,5,6-dibenzanthracene¹ 10 rats of the Wistar

* This investigation was aided by a fund for leukemia studies contributed anonymously.

¹ An extensive study in progress in this laboratory has shown that dibenzanthracene in benzol gives rise to tumors in chickens in a far shorter period than when the same amount of the chemical is administered in lard or chicken fat.

strain were injected in both groins with 0.5 cc. of a 3 per cent suspension of the chemical in benzol. This was followed 6 weeks later by a second injection in the same region. After another 6 weeks one rat was found to have a mass in the left groin, 1.2×0.7 cm. in size. A biopsy specimen showed a structure resembling a lymph node impregnated with crystals of dibenzanthracene. Two weeks later a mass was observed in the other groin. The blood count at this time showed 32,000 white cells per cu. mm., 91 per cent of which were mononuclears of the lymphoid type. A week later the white cell count had increased to 43,000 at which time the animal was killed and the blood collected from the heart to be used for an attempt at transmission.

The autopsy showed, besides the masses in the groins, marked enlargement of the lymph nodes of the axilla, neck, and peritoneum. There was no evidence of involvement of the liver, spleen, or other organs. Crystals of dibenzanthracene were found in both masses from the groins. Histologically the involved nodes were composed of closely packed lymphoid cells with disruption of the normal architecture.

Two other rats from the group developed local spindle cell sarcomas which were easily transmissible to other rats.

Transmission of leukemia.—The defibrinated blood from the original leukemia animal was injected into 7 Wistar rats of about 65 gm. in weight; 0.2 cc. in the subcutaneous tissue of the groin and 0.4 cc. intraperitoneally. The involved lymph nodes were finely minced, suspended in normal saline, and inoculated into 7 rats of the same strain. These received 0.2 cc. of the suspension subcutaneously and 0.6 cc. intraperitoneally. By the fourth week 3 of the 7 rats inoculated with blood, and 4 of the 7 injected with the cell suspension had developed typical leukemia which progressed to the death of the animals. This was characterized by a marked increase in the circulating lymphocytes and massive enlargement of the lymph nodes. The thymus in all of the affected animals was so large that it caused pressure on both the lungs and heart but, as with the original animal, there was no evidence of involvement of the other organs.

In subsequent generations both the number of takes and the severity of the disease has increased. In the first five transfers when young Wistar rats were used, the takes were from 60 to 80 per cent and the average length of life after inoculation was over 2 weeks, while in later generations the takes increased to between 90 to 100 per cent and the animals died in from 6 to 10 days.

Character of the disease.—The rise in circulating lymphocytes starts at about the 6th day after inoculation and progresses rapidly. A typical example of the changes in blood picture based on counts in 10 rats is shown in Fig. 1. In a number of animals surviving a little longer than the average, white counts of over 200,000 are not uncommon and in one instance it reached 260,000 cells per cu. mm.

Labored breathing from pressure of the thymus is noted early in the disease. There is usually a bloody discharge from the nose and about the eyes from small hemorrhages taking place in the mucosa of the nose and eyelids. At autopsy hemorrhages are noted in the involved lymph nodes, testicle, muscle, intestines, and subcutaneous tissue. All lymph nodes are greatly enlarged and the thymus is extensively involved (Fig. 2). The spleen may be slightly above the average size in some individuals but does not show a disruption of its normal structure or other evidence of involvement. The bone marrow is

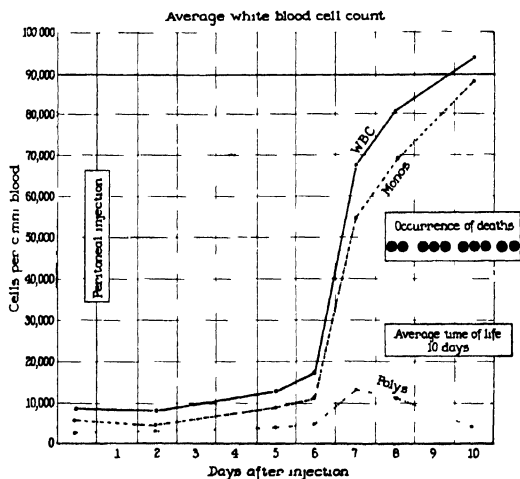


FIG. 1. The white blood cell count following intraperitoneal inoculation of leukemia cells based on the average counts in 10 rats. The black circles indicate the time of death of the individual animals.

largely replaced by lymphocytic infiltration but other organs show no evidence of involvement.

Production of lymphosarcoma.—As noted above in the first transmissions, the rats were inoculated both subcutaneously and intraperitoneally. The resultant disease was leukemia but in a few of these animals a very thin plaque of tissue was noted at the site of the subcutaneous injection. The fate of the leukemic blood inoculated subcutaneously was next investigated.

Experiments.—Blood from a leukemic animal of the 14th generation was injected subcutaneously in 4 rats and, as controls, intraperitoneally into 4 rats. By the 14th day, 3 of the 4 in the latter group died of leukemia while none of those inoculated subcutaneously showed the disease. However, 2 of the test group developed tumors at the site of inoculation which grew to a large size, and eventually caused the death of the animals. In the course of

subsequent generations some 929 rats have been inoculated subcutaneously with the tumor cells, leukemic blood, or involved lymph nodes. Of these, 522 have developed local tumors with no evidence of generalized disease. In later generations when there was an increase in malignancy, 7 rats inoculated subcutaneously developed local tumors and later showed leukemia. However, this continues to be of very rare occurrence. With the exception of these 7 animals the local nature of the disease produced by subcutaneous inoculation is indicated both by the fact that there is no increase in the circulating white cells of the blood and that the blood from these rats injected into normal rats



FIG. 2.—Involvement of the thymus and nodes of the neck in generalized leukemia resulting from intraperitoneal inoculations.

fails to produce either tumors or leukemia. A typical result of the blood changes in rats during the development of the transplanted lymphosarcoma is shown in Fig. 3 and in Table I.

The tumors resulting from the subcutaneous inoculation of leukemia cells grow to be very large, often attaining a size of 6×3 cm. by the 3rd week (Fig. 4). They differ from the usual transplanted tumor of the rat in that they actively invade the skin and underlying muscle (Fig. 5). Metastases to regional lymph nodes occur with some frequency. In 2 rats secondary growths were found in both eyes and another had a single eye involved. In these incidences the entire exposed part of the eyeball was covered by an opaque mass but at autopsy it was found not to involve either the eyeball or socket. The only point of attachment was by a thin pedicle to the inner canthus of the lids.

The subcutaneous tumors are firm and even in the very large ones there is little evidence of central necrosis. Histologically they are composed of closely packed, small round cells of fairly uniform size with scant stroma. Many mitotic figures are seen, particularly in the young tumors.

At intervals the ability of the tumor cells to cause leukemia has been tested. Even after being carried as a local tumor for many generations, when the cells are inoculated intraperitoneally the rats rapidly develop fatal leukemia. There have been only two cases of intraperitoneal tumors without generalized leukemia.

A summary of the transmission studies, with the number of animals utilized, is given in Fig. 6.

Results of inoculation of leukemic cells into the spleen.—The absence of any definite involvement of the spleen in the leukemia resulting from intraperitoneal

TABLE I
Blood Changes in Rats during the Development of Transplanted Lymphosarcoma

| | Inoculated intraperi- toneally, developed leukemia | Inoculated intraperi- toneally, no leukemia | Inoculated subcutaneously, local tumor |
|------------------------|----------------------------------------------------------------|------------------------------------------------------|----------------------------------------------|
| No of rats | 35 | 16 | 17 |
| Days after inoculation | 18 | 27 | 19 |
| Average W. B. C. | 76,100 | 9,280 | 10,600 |
| Small lymphocytes | 64% | 54% | 49.8% |
| Large lymphocytes | 29.8% | 13% | 9.2% |
| Polymorphonuclears | 6.0% | 33% | 40.9% |

inoculation in the presence of such general involvement has no evident explanation. It is generally considered that in the transmitted disease all of the leukemic cells are the descendants of those introduced by inoculation. To test the possibility that the spleen is relatively unsuitable for the development of the malignant cells, direct inoculations have been made into this organ.

Experiments.—The approximate size of spleens in the rats to be used was determined by direct measurement after bringing the spleen out through a small opening in the abdominal wall. The spleen was replaced leaving only the tip in the wound. About 0.5 cc. of leukemic blood was injected very slowly through a fine needle direct into the spleen pulp. In order to avoid leakage after withdrawal of the needle a gauze compress was held over the puncture till bleeding had ceased and the hole firmly blocked by clot.

Of the 16 rats so inoculated in three experiments, 8 were dead of the disease by the 6th day and only 2 survived as long as 10 days. The average length of life was 7.2 days. The blood showed a rapid rise in white cells, the average for the group being 60,000 per cu. mm., with 88 per cent lymphocytes. The

spleens increased from 2 to 4 times their original size and in several instances were over 5 cm. in length. The livers were enlarged, markedly congested, and showed extensive round cell infiltration. The mesenteric lymph nodes were involved but there was no increase in size of any of the other nodes and no evidence of involvement of the thymus in any of the animals.

With the great number of leukemic cells in the blood, a wide dissemination through the organs and tissues would be expected. Yet the development of the secondary deposits is strictly limited depending on the site of inoculation.

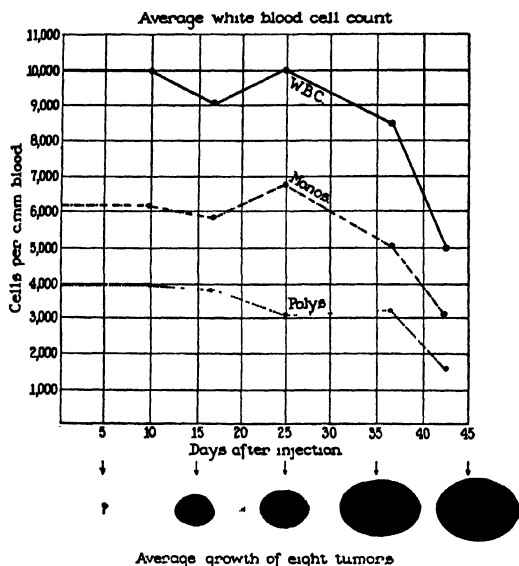


FIG. 3.—The white blood cell counts on 8 rats during the development of transplanted lymphosarcoma. The figures and measurements of tumors represent the average for the group.

The principal involvements following intraperitoneal injection are the superficial lymph nodes and the thymus; splenic inoculation results in deposits principally in the spleen and liver; and subcutaneous inoculation gives local tumors with only regional lymph nodes affected.

Survival of cells in frozen tissue.—It (5) has been shown that several mammalian transplanted tumors may be preserved in a frozen state over long periods. In the earlier passages three attempts were made to transmit the rat leukemia by blood and involved lymph nodes which had been kept in a frozen state for a period, but no results were obtained. However, in later passages tissue kept frozen for 14 days and then inoculated subcutaneously produced tumors in all of the animals. The tumors were somewhat slower

in starting but grew vigorously later. A spleen kept frozen for 21 days produced leukemia in all of the rats into which it was inoculated. Frozen blood has continued to give negative results.

Attempted transmission by cell-free material.—There have been no well authenticated examples of transmission of mammalian leukemia by cell-free material but for completeness attempts have been made with the present strain.



FIG. 4. Local lymphosarcoma from subcutaneous inoculations of blood from a rat with leukemia.

Experiments.—In five tests, the tumors or involved lymph nodes were frozen and desiccated *in vacuo*. The powdered desiccates taken up in water were inoculated into 69 rats. None of these developed either tumors or leukemia.

The disease is readily transmitted by the ascitic fluid from leukemic rats. This fluid collected from a number of such animals was filtered through paper and centrifuged sufficiently to bring down the cells. The supernatant fluid injected into 10 rats failed to induce the disease. The resuspended sediment containing cells produced leukemia in 6 out of 10 animals injected.

The possible presence of an agent in the serum of rats with leukemia has also been investigated. In two tests, 9 normal animals were inoculated with the



FIG. 5 —Extension of tumors shown in Fig 4 into the muscles of the leg

Transfer of an induced lymphatic leukemia

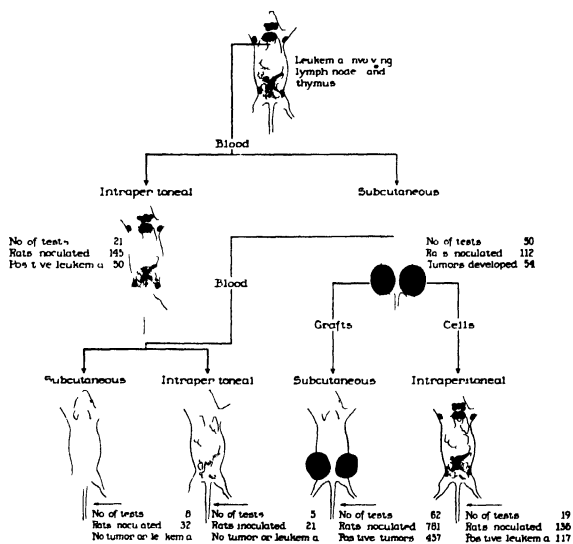


FIG. 6 —Transfer of an induced lymphatic leukemia

serum and 11 with the sedimented blood cells from rats with advanced leukemia. None of those with the serum showed any reaction while 7 injected with the blood cells developed the disease.

Failure of transmission to other species.—Some 60 mice have been inoculated with blood from rats with leukemia either subcutaneously or intraperitoneally but none of them showed any evidence of tumors or leukemia.

Using Green's method (5), rat leukemic cells were inoculated into the anterior chamber of the eye of 4 rabbits and 8 guinea pigs. There was no growth observed at any time and no surviving cells could be found at the end of 4 months.

Grafts of the rat lymphosarcoma were inoculated into the right frontal lobe of the brain of 12 mice and 2 rabbits (11). No evidence of growth or survival of the cells could be found in any of the animals. A number of other attempts have been made to induce leukemia in rats with carcinogenic chemicals but so far this has succeeded only in the one instance reported above. Further attempts are in progress.

DISCUSSION

The close relationship between leukemia and malignant tumors is so generally conceded that it needs no further emphasis. There are now a number of examples of leukemias induced in mice by the same chemicals which are known to induce both carcinoma and sarcoma. In certain strains of mice the type of response to the carcinogenic chemical is influenced by the genetic tendency of the strain to develop certain types of spontaneous tumors. For example, Lynch and others have shown that the development of induced lung tumors in any given strain varies directly with the degree of normal tendency of the various strains to develop spontaneous lung tumors. Apparently the same principle holds for leukemia in mice as in most of the strains in which leukemia has been induced, the disease also is known to occur spontaneously. It is suggested that with limitations the carcinogenic agents accentuate the tendency to develop certain types of tumor or leukemia.

A point of interest in the present investigation is that leukemia has been induced in a species in which spontaneous leukemia is probably of very rare occurrence as no cases are on record. Lymphoid tumors have been observed but even these are rare in the strain of rats utilized for these studies according to the only available information.

There is a clear-cut variation in the manifestation of the transplanted disease, dependent on the site of inoculation. Why the spleen and liver should escape involvement under one condition and the lymph nodes and thymus escape under another, when the blood stream is teeming with the malignant cells, is not immediately explainable. Nor is it evident why the lymphosarcoma resulting from subcutaneous inoculation which actively invades all the surround-

ing tissues including metastasis to regional lymph nodes so rarely gives rise to generalized leukemia.

This first easily transferable typical leukemia in a larger mammal gives new material for investigation of a type of malignant disease.

SUMMARY

A rat which had been injected in both groins with 1,2,5,6-dibenzanthracene in benzol developed typical lymphatic leukemia. The first lesions were noted in the regional lymph nodes where crystals of the agent were found embedded in the tissue. It has proved possible to transfer the disease to other rats but the manifestations of the disease are varied depending on the site of injection of leukemia cells. When given intraperitoneally, the disease is manifested in the blood stream with involvement of only the lymph nodes and thymus. Subcutaneous inoculations with either blood cells or involved nodes with few exceptions produced local lymphosarcomas spreading only as far as the regional lymph nodes. Inoculation into the spleen produces leukemia with the principal manifestation in the spleen and liver leaving the superficial nodes and thymus untouched.

The transmitted disease is described.

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INHERITANCE IN GUINEA PIGS OF THE SUSCEPTIBILITY TO SKIN SENSITIZATION WITH SIMPLE CHEMICAL COMPOUNDS

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In studies on skin hypersensitivity induced by simple substances, it has frequently been noticed by various workers that an identical treatment would lead to different degrees of sensitiveness within groups of guinea pigs even when kept under uniform conditions of diet and housing (*cf.* 1, 2); similar observations have been made in experimental sensitization of human beings with simple chemical compounds, for instance by Sulzberger and coworkers (3, 4; *cf.* 5, 6). The question arose whether the variations observed in the animals bespeak hereditary properties, as one might well surmise, apart from other factors, but thus far there has been no definite proof. For the investigation of this matter we bred guinea pigs chosen for high (and low) susceptibility to experimental sensitization and studied susceptibility in the progeny; earlier results were reported briefly (7). The substance selected for sensitization was 2:4 dinitrochlorobenzene, which had proved to be a very suitable compound for experiments on drug allergy of the contact dermatitis type (1, 8). The experiments were devised to exclude the influence of feeding and seasonal factors (*cf.* 9, 10), which are therefore outside of the scope of this paper.

A particular influence of hereditary constitution is well known in certain forms of human allergy (hay fever, asthma)¹ (Cooke and Vander Veer (12), Coca and Cooke (13), Spain and Cooke (14)). Apparently this has not so far been induced experimentally in human beings in contradistinction to drug allergy in which with certain incitants sensitization is successful in almost every case (3, 15; *cf.* 16).

Various investigations have been conducted with laboratory animals which demonstrate inheritance of such qualities as the capacity to produce antibodies, a predisposition to anaphylactic effects, and resistance to infection. Inbred families of guinea pigs were studied by Lewis *et al.* and found to differ as regards ability to engender antibodies (17) and in resistance to tuberculous infection (18). Furthermore Lurie (19, 20) has found

¹ A genetic analysis is offered in a paper by Wiener *et al.* (11).

in inbred strains of rabbits marked differences towards infection by tubercle bacilli, and he was able to demonstrate several somatic characteristics associated with resistance or susceptibility. Well known are the extensive studies by Webster (21, 22), which have resulted in the establishment of strains of mice of high and low susceptibilities to bacterial infections, and to certain viruses.

An interesting report dealing with individual differences among guinea pigs in the amount of diphtheria toxin necessary for antitoxic immunization has been presented by Prigge (23): while the ratio of immunizing dose between the extremes among guinea pigs purchased for the Frankfort Institute had the surprisingly high value of 1:32,000, the individual differences with inbred strains, on the other hand, were less pronounced, being only 1:25 for one of these.

EXPERIMENTAL

Selection of Parents.—Male and female albino guinea pigs were sensitized and tested with 2:4 dinitrochlorobenzene² as described below, in order to obtain high and low reactors with which to set up breeding colonies for study of the inheritance of the susceptibility to sensitization in the progeny. The animals for starting both the colonies originated chiefly from two sources, one being the breeding room at the Institute, with a few added later from a third stock. There were several complicating factors in the choice of initial breeders. A selection among low reactor animals is limited because of the scarcity in common stocks of albino guinea pigs refractory to sensitization with dinitrochlorobenzene, a potent sensitizer (24); this is discussed further on. Then female guinea pigs exhibit in general a lower level of reactivity than males, making uncertain the estimation of female reactors of the lowest grade. Finally, a rather long sensitization procedure may succeed in disguising mediocrity by raising the sensitivity to an apparently acceptable level. Largely for these reasons it was found necessary, in addition to selecting the individuals on the basis of their own attained sensitization, to retain them in the breeding colony only after a trial mating and determination of the sensitization level of the offspring. Examples are given in Fig. 1. (The offspring of unsuitable breeders were likewise excluded.) This procedure once established, it proved feasible to erect colonies in which reactivities of the offspring were usually predictable (see Figs. 2 to 4).

To start the high reactor colony, 3 males (selected from 25 sensitized guinea pigs) and 6 females (chosen from 20) were employed; on the basis of the progeny test one male and one female were accepted. The data for this selection are shown in part in Fig. 1, A, B, C. Among the six litters resulting when 3 females (Nos. 481, 5, 8) were mated in turn with the same 2 males (Nos. 343 and 34), there was considerable variability as regards capacity for sensitization, ranging all the way from resistant to highly susceptible litters. The choice of Nos. 34 and 8 (Fig. 1, C) as initial breeders appeared expedient. Their 3 sons (the mother being no longer available) were bred with a new selection of females (8 picked from 63 sensitized individuals of which 5 were retained after an initial trial mating, and 2, themselves high reactors, transferred from the low reactor colony). By this means and through suitable matings involving 19 females and 21 males produced in the colony (in part backcrosses of offspring to parent and brother-sister inbreeding),

² Some animals used in the first matings had been sensitized with 2:4:6 trinitrochlorobenzene (picryl chloride) instead.

there were secured and tested 112 descendants representing 4 generations, after which pen-inbreeding using tested males was introduced. Some of the matings with less closely related males were made during the several months necessary for the maturing and testing of particular litters.

For the low reactor colony similar methods were followed (*cf.* Fig. 1, *D* and *E*), but consideration was given to the result of the longer course of injections in the progeny as

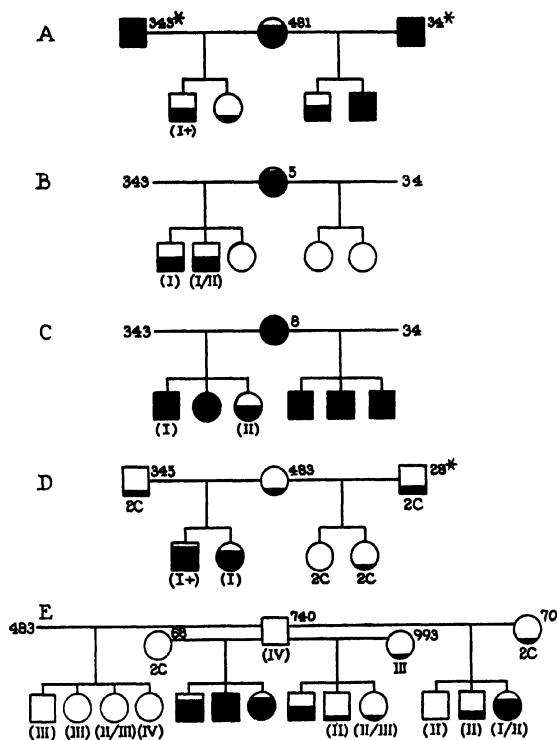


FIG. 1. Typical selection for breeding stock among sensitized animals by means of progeny test. Of the parents, those marked with an asterisk were sensitized with picryl chloride instead of dinitrochlorobenzene. All offspring were tested with the latter substance.

The degree of skin reactivity is indicated by the extent of shading, described on pages 180-181; circles and squares designate females and males respectively. The symbols show the level of sensitivity after the brief course of treatment (except that 2C signifies test after 14 injections); the sensitivity levels after the second course, when determined, is indicated by Roman numerals.

Experiences with high reacting animals are given in *A*, *B*, and *C*, while trial matings among the low reactors are shown in *D* and *E*.

well, exemplified by the choice of Nos. 483 and 28 (Fig. 1, *D*) and the rejection of Nos. 345 ♂, 68 ♀, and 70 ♀. 5 males (picked from 52) and 18 females (chosen from 83) were introduced; 3 of the males and 10 of the females were kept after trial *via* the progeny test. From these there have been 110 descendants, representing 4 family generations, produced by the initial matings and by the breeding of 17 female and 10 male offspring.

Maintenance of the Colonies.—The animals were kept uniformly on a "dry diet," receiving hay, oats, and liberal amounts of chopped cabbage daily. Not more than 4 sows were housed with one buck; the females were removed to individual cages in the 2nd month of pregnancy, and offspring were segregated by sexes at an age of about 4 weeks. The guinea pigs remained in good health, without intercurrent infections.³ Incidentally animals in the colony, skin-tested with Moen's antigen to detect carriers of streptococci causing epizootic lymphadenitis (25), were found not to react.

Sensitization Procedures.—Testing of the progeny was commenced when the animals weighed between 340 and 400 gm., at the most 500 gm.; the females were not mated before the final test results were obtained. Adequate numbers of male and female offspring from both the high and low reactor colonies were assembled for each sensitizing course, thus ensuring comparative testing, and cancelling possible seasonal or other variations due to external influences on the selection of animals for breeding.

For sensitizing, injections of 1/400 mg. dinitrochlorobenzene in 0.1 cc. saline were made into the skin of the back; solutions of the proper concentration were prepared freshly each time by diluting in saline an alcoholic 0.3 per cent solution of the recrystallized commercial preparation. It was soon learned that fewer injections of the incitant were necessary to sensitize animals of the "high reactor" class. All progeny therefore were first given 4 injections, twice weekly for 2 weeks, and were tested 2 or 3 weeks after the last injection: this first, brief course assisted the establishment of the "good" colony by indicating the superior reactors. Next the animals received a further course of 10 daily injections, and were tested again 3 weeks after the final injection. This longer treatment served principally to detect animals of intermediate capacity for sensitization and to exclude them from the low reactor colony;⁴ at times the reactivity was found to have declined somewhat following the longer treatment (7 out of 66 animals in the susceptible colony, 13 out of 88 in the resistant colony).

For testing the sensitivity, 1 drop of a 1 per cent solution of 2:4 dinitrochlorobenzene in olive oil was spread on the belly, after clipping the hair, over an area of 8 to 10 sq. cm.; fresh sites were used for each test and normal animals were included as controls. The reactions were examined on the next day, following use of a depilatory 2 or 3 hours before. All males in an experiment, and likewise the females separately, were put together and then sorted out comparatively into four primary classes of reactors (negative up to high reactors) and the ratings were recorded without knowledge of their origins. The intensity of the reactions and the assigned grades were as follows: pink, commonly slightly elevated (I); pale pink (II); faint pink (III); negative or at most a minimal reaction (IV). Intergrades, as I/II, also were definitely recognizable. The symbols in Figs. 1 to 4 indicate the gradings by the relative amount of shading, I being shown as

³ An exception was the loss of 261 ♂, 262 ♀, in the more resistant colony.

⁴ Likewise Webster (21, 22) originally used different doses of living bacteria in the establishment of his susceptible and resistant mice.

entirely black, IV as white, II/III as half black, and so on. A special symbol (see Figs. 2, 4) is assigned to unusually superior reactors which may be known as I+, namely those showing bright pink, often somewhat swollen, test sites. Readings after the first and second courses are indicated by an arrow, e.g., II \rightarrow I.

Since information was desired about the responses of the guinea pigs in the colonies to a second type of incitant, some of the lots following the terminal dinitrochlorobenzene testing were sensitized to poison ivy (26, 27; cf. 28), using poison ivy extract (Lederle),⁵ a 13 per cent solution in acetone of extractives from *Rhus toxicodendron radicans*. To effect sensitization, 1 drop of a 1:5 dilution in alcohol was allowed to fall on the lumbar region of the back and was spread with a glass rod over an area about 15 mm. in diameter. On the 4th day the ivy was removed by cotton pledgets soaked in acetone. The test for cutaneous sensitivity, described in detail elsewhere (28), was made between the 10th and 14th days, single drops of dilutions in alcohol being applied to various areas of the skin. The reactions were recorded at 24 and 48 hours, and ratings assigned according to the lowest effective dilution and the intensity of the reactions. (These methods were used also in some breeding experiments undertaken with respect to susceptibility towards ivy sensitization.)

As regards the selection for high reacting animals, the responses of the progeny to sensitization with dinitrochlorobenzene are presented in Fig. 2⁶ and are set forth summarily in Table I. The symbols indicate the skin reactivities developed by the "brief" course of 4 injections, with only 0.01 mg. in all of the incitant. It may be remarked at the outset that skin tests on some of the young made within 10 days after birth or when 2 to 3 months old were negative, proving that we were not here dealing with direct acquisition of hypersensitivity⁷ from the sensitized mothers; furthermore, the onset of skin sensitivity to the successive injections has not been different in these animals from that observed with bought guinea pigs. Of the males born in the colony, 68 per cent became reactors of grade I following the brief course, and by further treatment with the incitant the proportion of animals in grade I was increased to some extent, e.g. from 66.6 to 78 per cent in the 36 so treated (Table I).

⁵ This was supplied through the courtesy of Dr. Arthur F. Coca.

⁶ For graphical reasons, there have been omitted from Fig. 2 the following crosses: 387 $\sigma \times$ 378 φ , giving a daughter (II \rightarrow I); the latter \times father 387, giving one I + son, and one son and one daughter of grade I \rightarrow I +; 681 $\sigma \times$ 197 φ , producing 556 σ (I) and a daughter of grade II; and 556 $\sigma \times$ mother 197, giving a daughter of grade III \rightarrow II; 387 $\sigma \times$ 197 φ , producing a daughter (I/II \rightarrow I). There were two instances of fertilization due to error; in the case of 94 φ , impregnation was known to have occurred in a cage containing 3 males, all of grade I.

⁷ This is also evidenced by the general correlation between the degrees of hypersensitivity induced by dinitrochlorobenzene and poison ivy extract, discussed further on, which was seen in animals whose ancestors had not been treated with ivy extract.

In detail, among these the retest after the second course gave the following result: with 13 there was no change in classification; slight changes, as I/II \rightarrow I, occurred with 14; there was a substantial increase in the grading of 6; and with 3 the early high sensitivity (I) had declined to II, II, and III respectively.

Of the 45 females, 42.2 per cent were of grade I after the short treatment, and following another 10 injections in 30 of the animals there was a pro-

TABLE I

Sensitivity Responses of Progeny in the Susceptible and the Low Reactor (More Resistant) Colonies

| Grade of response | Susceptible colony | | | | Low reactor colony | | | |
|-------------------|----------------------------|-------------|-------------------|------------------|----------------------------|-------------|-------------------|------------------|
| | Animals given both courses | | Brief course only | Long course only | Animals given both courses | | Brief course only | Long course only |
| | Brief course | Long course | | | Brief course | Long course | | |
| | Males | | | | | | | |
| I+ | 5 | 7 | 6 | | 1 | | | |
| I | 19 | 21 | 15 | 1 | 2 | 6 | | |
| I/II | 3 | 5 | 2 | | 1 | 5 | | |
| II | 3 | 2 | 4 | | 2 | 3 | | 1 |
| II/III | 4 | | | | 4 | 11 | 3 | |
| III | 2 | 1 | 1 | | 9 | 6 | | 1 |
| III/IV | | | | | 7 | 2 | | |
| IV | | | 2 | | 16 | 7 | 7 | |
| | Females | | | | | | | |
| I+ | 1 | 8 | 4 | | 6 | | | |
| I | 10 | 14 | 4 | | 1 | 5 | | |
| I/II | 2 | 2 | 2 | | 2 | 1 | | |
| II | 6 | 2 | 2 | | 2 | 8 | | |
| II/III | 3 | 2 | 1 | | 5 | 4 | 2 | |
| III | 4 | | 1 | | 9 | 8 | 3 | |
| III/IV | 1 | | 1 | | 9 | 4 | 1 | 2 |
| IV | 3 | 2 | | | 19 | 11 | 1 | 1 |

nounced general rise in sensitivity, the proportion in grade I increasing from 36.6 to 73.3 per cent, and in effect obliterating the sex difference.

Due to limited options, probably connected with the lower susceptibility of females, it proved necessary to use as breeders a number of females which were not especially superior after the brief course: while 18 out of the 21 bucks were early grade I reactors, this was true of only 14 out of 27 females. It may be supposed, and inspection of the applicable data would suggest, that an adequate choice among females would have improved the status of the colony. For this purpose, the mothers and their offspring are assigned to three classes in the appended tabulation on the basis of response to the brief sensitization course, uniformity among the fathers as regards early high sensitization appearing to permit an approximation of this sort.

| Early rating of sows | No. of sows | Classification of offspring following brief course | | |
|----------------------|-------------|----------------------------------------------------|------|--------------|
| | | I | I/II | II and below |
| I | 12 | 25 (9 I+) | 2 | 7 |
| I/II | 6 | 17 (2 I+) | 3 | 11 |
| II and below | 8 | 15 (2 I+) | 2 | 12 |

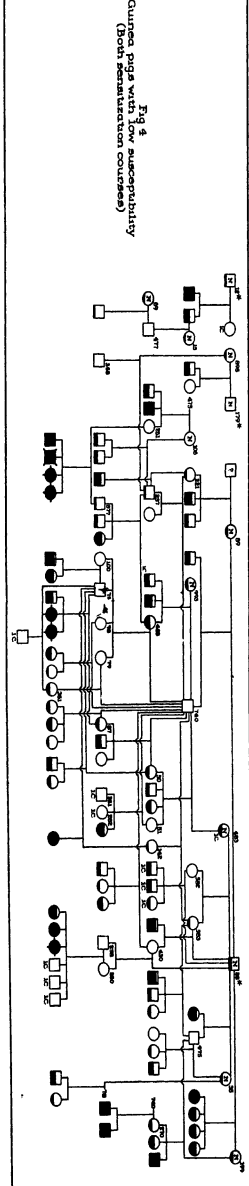
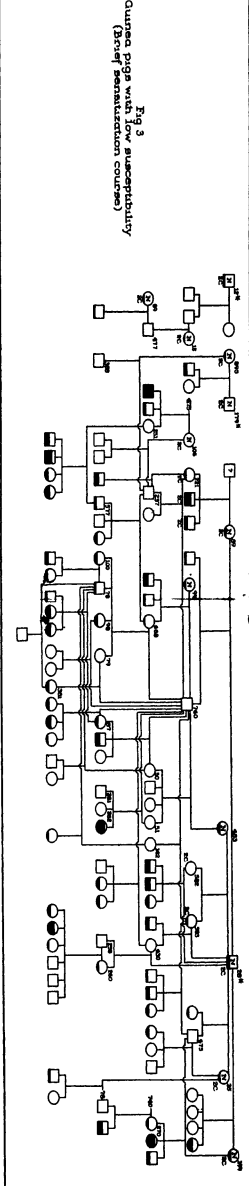
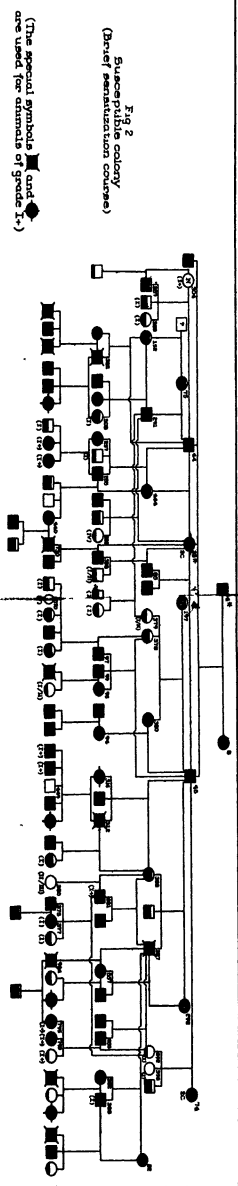
It appears that nearly three-fourths (73.5 per cent) of the offspring of grade I mothers are themselves of grade I, as compared with about 55 and 52 per cent of the offspring of mothers of the two lower categories respectively. And likewise suggestive, perhaps, is the proportion of animals exhibiting unusually brilliant reactions (designated as I +) born to the females of early high *versus* those of intermediate reactivity.

The genetic evidence of Fig. 2, by itself, suggests forcefully the segregation of factors influencing susceptibility to sensitization. Apart from the animals in class I, there is a variety of lesser grades, a common type being II/III \rightarrow I or I/II. Familial tendencies in respect to this "delayed" sensitization are probably shown by 393 ♀ (IV \rightarrow I), its sister 392 (III/IV \rightarrow I) and 2 daughters of the latter by different males, 681 ♂ and 253 ♂, the ratings in question being II/III \rightarrow I and III \rightarrow I + respectively, and in the extreme case by 304 ♀ (IV \rightarrow I+) and its three offspring by the grade I reactor 44 ♂ (see page 188). 2 animals given the full 14 injections responded slightly or not at all (268 ♀, III \rightarrow IV; 370 ♀, IV \rightarrow IV).

The following instances may be cited in which an inhomogeneous condition in the parents was not revealed fully at least by their phenotype testing but by segregation in the progeny. The mating of son and daughter from the backcross of 387 ♂ with the latter's mother, these 4 all being of early grade I, gave 2 daughters with unlike reactions, one of grade I +, the other II/III. Sharp contrasts among the offspring occurred also from the brother-sister cross 363 \times 362 (*cf.* 360 ♀ born to 681 ♂ \times 388 ♀). Again, from the backcross of 464 ♀ with its father 3 offspring were raised, one male a III, the other brother and sister each of grade I; thereupon a mating of the high reactor brother (255) with its sister gave rise to a son of grade II/III and 2 grade I females, while a backcross to its mother produced 2 males and a female which were II, IV, and I respectively. Then matings of 97 (I) with its mother 378 (II) gave 5 offspring which fell into several types, IV \rightarrow IV (♀), II/III \rightarrow I (♂), II \rightarrow I (2 ♀), I \rightarrow I (♂). A similar case of one offspring conspicuously resistant to sensitization in contrast to its brothers and sisters is afforded by the 5 progeny from the brother-sister mating 312 (I+) \times 314 (I+).

In two families, probably because of small numbers, only grade I reactors were encountered (44 ♂ \times 152 ♀, 46 ♂ \times daughter 380).

Surveying the results obtained with the strain of high susceptibility, it has been possible to secure a marked improvement over usual guinea pig stocks. After the second generation, the regularity of the response to



Figs. 2 to 4. The levels of sensitivity are indicated by relative extent of shading (grades 180-181), varying from symbols without shading (Grade IV, resistant animals) to those entirely black (Grade I, superior reactors); in addition, the special devices noted above are used to designate particularly brilliant reactors among the males and females respectively.

The symbols show the degree of sensitivity attained by the stated procedure, *genet*, that if data are wanting 1C and 2C signify the result after the first or second courses of injections respectively. In Fig. 2 the grade after the second course, wherever significant, is given in Roman numeral subscripts. An identification number without accompanying symbol indicates that the animal in question appears elsewhere in the figure, usually in the same horizontal line. N = animals introduced into the colonies; some of these (*) had been sensitized with plicyl chloride.

experimental sensitization was not considerably increased by such selective matings as were practicable, the lack of a sufficient number of highly susceptible females probably contributing to this. But it would appear that the occurrence of animals exhibiting especially brilliant reactions (I+), often after only the brief course of injections, is increasing in frequency with continued selection of parents (Table II). Among the females of the fourth generation, for instance, 10 out of 23 were superior reactors at some time during the sensitization, as compared with 2 out of 12 in the third generation. Again, in preliminary tests, titrations by applying to the skin decreasing concentrations of the incitant have indicated that, whereas animals from unselected stocks sensitized by comparable procedure and picked as good reactors seldom reacted to a 1/25 per cent solution in olive oil, definite

TABLE II

Occurrence of Animals of Especially High Reactivity (Grade I+)

The number of I+ animals is shown first, followed by the total number submitted to the given sensitization procedure, *i.e.*, one or two courses.

| Generation | Males | | Females | |
|----------------|-----------------------|---------------------------|-----------------------|---------------------------|
| | I+ after brief course | I+ developed by 2d course | I+ after brief course | I+ developed by 2d course |
| F ₂ | 1/8 | 0/4 | 0/9 | 0/4 |
| F ₃ | 2/20 | 1/18 | 1/12 | 1/10 |
| F ₄ | 8/28 | 3/11 | 4/23 | 6/14 |

reactions to this concentration have been observed with about two-thirds of the limited numbers of high reactor progeny tested.

It may be mentioned that also by another mode of sensitization, namely repeated applications to the skin of alcoholic solutions of the incitant, animals produced in the high reactor colony have shown themselves superior to other stocks.

In sharp contrast to the high reactor colony are the results with the strain bred for low susceptibility. The reactivity after the brief course of injections is shown in Fig. 3⁸ and the effects seen after the second course are given separately as Fig. 4. The evident disparity between Figs. 2 and 3 is not extinguished even by the longer treatment, although the latter nearly always brought about an increase in reactivity. From the data in Table I, 41 per cent of the offspring raised in the colony belonged in grade IV after the short course, and 3 out of 4 (77.1 per cent) were grade III and below,

⁸ The data in Figs. 3 and 4 do not show some lines which were abandoned in the third generation because of too few offspring.

that is, worthless for most experimental work in drug allergy; there were no significant sex differences. When further injections of the incitant were given, only 17 per cent of the males and 23.4 per cent of the females were of grade IV, although 36.6 and 49 per cent respectively were still not higher than grade III. It should be added that, while a few guinea pigs in average albino stocks appear to be refractory after 10 to 15 daily injections of 2:4 dinitrochlorobenzene, it is highly doubtful whether an absolute resistance will be encountered in any guinea pig under more intensive treatment, for instance repeated applications of oil or alcohol solutions to the skin.⁹ The lowest category used in the charts, grade IV, does not signify entire resistance, for some trifling hyperreactivity to the daily injections was noted in nearly all these animals during the second course; however, in the final contact test after the rest period the animals could not be distinguished surely from those of the normal controls which showed some slight skin irritation to the chemical.

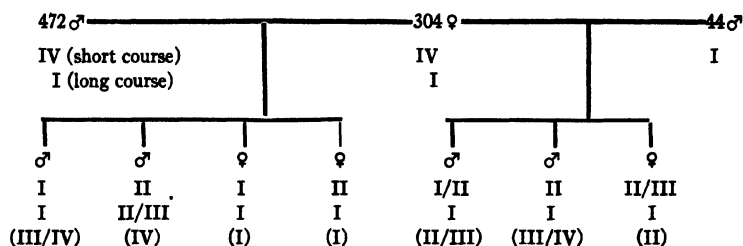
The greater difficulty in breeding for low susceptibility to sensitization was evident in the earliest attempts. Contributing complications in the selection of the breeding stock were mentioned above (page 178); probably as a consequence there was less initial homogeneity than among the high reactor stock. As regards improvement of the colony, a choice of "continuing" low reactors among the progeny, particularly brothers and sisters, was quite limited. Of the 7 male offspring suitable for breeding, 2 had given slight reactions after the brief course, while among 11 females attempts to mate 4 were fruitless, the breeding of 2 others was scarcely productive, and the remaining 5 included one which had been early grade III and 2 that were early grade II/III.

Since the tendency for resistant animals to produce susceptible offspring is much greater than the reverse, it is probable genetically that the chief factors contributing to resistance are dominant over those favoring susceptibility to experimental sensitization. A few instances of "early resistant" animals from the mating of susceptibles (Fig. 2, *e.g.* 449 ♂, and Fig. 1, *B*) appear, however, to be out of line with a simple presumption that "resistance is dominant over susceptibility." Indeed, the data otherwise suggest that a plurality of factors controls susceptibility and resistance, in particular because of the occurrence of a rather large number of patterns, instead of a few, in the acquired levels of sensitivity. Other than this, if

⁹ Of some 234 albino guinea pigs sensitized in this manner, by only 5 to 7 daily applications, not more than 4 appeared to be refractory. This would then be analogous to the situation with *Primula dermatitis* (16), which is acquired by nearly every individual upon intensive treatment with extractives of the plant, although under natural conditions of contact marked individual differences in the capacity for sensitization are evident.

one considers only the following broad types: early susceptible, delayed susceptible (as III \rightarrow I), and continuing resistant (IV \rightarrow IV), it is not probable that a single pair of allelomorphs is adequate to explain the sensitization behavior of the progeny, *e.g.* in the line: father 28 \times 583 \varnothing , 28 \times 430 \varnothing , brother 258 \times 260 \varnothing (Figs. 3, 4). The intricacy is further to be seen from the various matings of 740 σ and of 78 σ (and from the discrepancies, mentioned below, between the sensitivities caused by use of two incitants). It will be noted in this colony that throughout the breeding of guinea pigs selected for their relatively high resistance, we did not meet with any positive instance of a "homozygous" animal as demonstrable by the testing of its progeny (although this well could be a consequence of the limited combinations of parents employed).

Of interest in connection with the genetic situation are the results of mating two delayed reactors of unusual behavior, and the female also with an early susceptible male: the gradings after the brief and second courses are given, and below, within parentheses, the sensitivity developed to poison ivy following treatment of the progeny with the latter.



Crosses involving members of the susceptible and resistant colonies were not undertaken since the latter group was not sufficiently uniform.

The essential difference between the two colonies and the evident segregations in the offspring indicate a genetic basis for susceptibility to drug allergy in the guinea pig, albeit a complicated one, and this is supported by the finding (after our breeding experiments were well along) of distinctly different levels of susceptibility among the albino stocks offered by various breeders. Among these instances may be mentioned one stock characterized by a low to moderate susceptibility to sensitization by intracutaneous injections of dinitrochlorobenzene and another which was distinctly inferior to other animals in our experience as regards skin sensitization to picryl chloride following intraperitoneal injection of this incitant in conjunction with dead tubercle bacilli (29).

We also conducted analogous breeding experiments with animals chosen

on the basis of high and low sensitivities developed to poison ivy (see page 181) instead of to dinitrochlorobenzene. The number of progeny examined was not large, but the results tended to show that there is an hereditary basis for susceptibility in this case also.

The question arose whether the difference between the two colonies is actually one of sensitization capacity or simply a lower or higher resistance to the primary toxicity of the incitant (upon which acquired sensitivities, possibly not unlike in degree, would be superimposed). For this purpose, 4 males and 4 females from each colony were assembled and, prior to the regular sensitization course, the skin reactions (primary toxicity) to different concentrations of dinitrochlorobenzene (1.5 to 0.75 per cent in alcohol, 10 to 1 per cent in olive oil) were determined. The brief intracutaneous sensitization course was then given, and after a rest period of 2 weeks the animals were tested with a 1 per cent solution in olive oil (which is seldom irritating and which had not sufficed to differentiate any of these same animals before sensitization).

There was, in fact, a difference between members of the two colonies, particularly among the females, with respect to the primary toxicity of dinitrochlorobenzene, skin irritations being produced in members of the high reactor colony by about one-half to one-third the concentration giving rise to the same degree of irritation in the low reactor colony. The significance of this as an explanation of the difference between the two colonies apparently can be discounted, however, because there was no consistent parallelism in the individual cases between primary toxicity and the level of hypersensitivity attained by treatment with the incitant. For instance, among the males from the high reactor colony the one exhibiting the greatest primary toxicity happened to be a poor reactor, and among the females from the low reactor colony the one least irritated by the incitant developed the highest sensitivity of the group. Another potent argument for actual differences in degrees of specific sensitivity is the frequent occurrence in the susceptible group of reactions to 1/25 per cent solutions of the incitant in olive oil, mentioned above, as compared with the poor responses to the 1 per cent test solution among members of the low reactor colony, a ratio much greater than the one between the concentrations producing irritation in non-sensitized animals of the two colonies. Nevertheless, this somatic difference between members of the two colonies may have a bearing on the multiplicity of sensitization patterns observed.

With a number of guinea pigs (100 progeny from the susceptible and resistant colonies, 37 offspring of animals selected according to their response to poison ivy extract, and 27 other guinea pigs), sensitization courses were

given successively with the two incitants to compare the respective responses. There was, in fact, commonly a parallelism between the degrees of sensitivity acquired to the two types of incitant, as stated for human beings by Wedroff and Dolgoff (5), yet discrepancies have appeared: these have been chiefly in the direction of a dinitrochlorobenzene sensitivity higher than that towards ivy, but there were at least 20 clear instances of the reverse.

Experiments of this sort should be extended, perhaps with the breeding of animals having unequal sensitivities to two incitants, and using methods of sensitization chosen to allow simultaneous testing with the respective chemicals.

The relationship observed will depend obviously upon the intensity of treatment with each sensitizer. For instance, the correlation was higher if the response to the ivy extract was compared with the dinitrochlorobenzene brief course. The experiences with 46 animals from the susceptible colony and 54 from the more resistant colony may be cited: after the brief course, 52 per cent of the offspring showed close agreement between the responses to the two incitants (*e.g.*, variations of not greater order than II *versus* I/II, IV *versus* III/IV, etc.), and after further dinitrochlorobenzene treatment in the second course 42 per cent were still in close correspondence.

Other data bearing on discrepancies may be cited.¹⁰ 2 males from the DNCl (dinitrochlorobenzene) stock which had scarcely responded to picryl chloride after a course of 13 intracutaneous injections of this substance were then given the brief course of DNCl injections; both became definitely sensitive to the second incitant (I/II and III respectively), and the discrepancy was confirmed upon retesting with picryl chloride and dinitrochlorobenzene simultaneously. Again, in one experiment 20 guinea pigs from common stocks were sensitized concurrently with both ivy and dinitrochlorobenzene, the latter being here used as a 5 per cent solution in olive oil applied to the skin daily for 4 days, and were tested with the two substances at the same time. The sensitization levels with the two incitants corresponded in 15 animals; of the others, one was moderately sensitive to DNCl but quite high with ivy while two others showed the opposite relationship, another was high towards DNCl and very weak towards ivy, and still another exhibited rather good DNCl and low-to-moderate ivy responses.

COMMENT

The establishment by controlled breeding of two colonies of guinea pigs which differ significantly in the degree of sensitivity attained following the same sensitizing procedure demonstrates the existence of variations of an hereditary nature in the capacity for sensitization. The difference between the two colonies was striking in that the one strain gave in the great majority

¹⁰ Compare the ivy and dinitrochlorobenzene responses in the tabulation on page 188.

of cases uniformly intense reactions after a brief course of intracutaneous injections with a total of 0.01 mg. substance, while the other responded almost regularly to an even greater number of injections with only a low grade sensitivity; however, these animals were not entirely refractory. In the latter group the uniformity was much less pronounced than among the good reactors, that is to say the offspring of poor reactors not seldom proved to be unequal, some individuals in a litter frequently exhibiting stronger effects than either parent. It might well be, however, that continuation of selective inbreeding would eventually lead to a more uniform strain of poorly reacting animals. It may be remarked that also Webster in the studies referred to above had greater difficulty in establishing a strain of mice with high resistance to infection with mouse typhoid than a highly susceptible strain.

Suggestive evidence for inherited differences as regards drug sensitization comes forth from experiences with guinea pig strains procured from different breeders. It appeared that guinea pigs (albinos) from some sources responded so poorly to sensitization that they were unsuitable for our experimental purposes, even after being kept for some time under our regimen.

The experiments were not carried far enough, and the situation is as yet too complicated, to offer a genetic analysis. Also it was not feasible to undertake repeated matings between parents of different types to obtain information about the ratios in the offspring. Several features, particularly the fact that the sensitivities do not fall into a few sharply discrete grades but show continuous transitions, would appear to contravene a supposition of a single pair of genetic factors.

Breeding experiments with parents selected for their reactivity to poison ivy, although made only on a small scale, tend to show that here again the degree of susceptibility is hereditary and that good and poor colonies can be established. This raises the point whether animals of different susceptibilities to sensitization with one simple substance will show the same behavior towards a different compound, that is, whether one deals with a susceptibility that is general or one varying to some extent according to the substances tested. From experiments in which animals were sensitized in succession to dinitrochlorobenzene and poison ivy, it would seem that there is roughly an accordance between the sensitivities developed to the two compounds (*cf.* 5) but there were several instances of discrepancy one way or the other. If further experience were confirmatory, this likewise would affirm the complexity of the hereditary basis underlying drug allergy. Observations indicating a degree of specificity in experimental sensitization of human beings have recently been reported (30).

It may be added that the possession of guinea pigs highly and uniformly susceptible to sensitization should be of value for experimental work in this field.

SUMMARY

It has proved possible to set up lines of guinea pigs of significantly different susceptibilities towards a compound of simple structure, namely 2:4 dinitrochlorobenzene. This provides direct evidence that the type of sensitization under discussion is influenced by heredity.

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IMMUNIZING CAPACITY OF VIRUS OF EASTERN EQUINE ENCEPHALOMYELITIS INACTIVATED BY ULTRAVIOLET LIGHT

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The inactivating effect of ultraviolet light on viruses as well as bacteria has been observed repeatedly. That viruses so inactivated may be effective as immunizing antigens has already been shown for at least 2 viruses capable of causing disease in man. With a vaccine prepared by irradiating mouse-brain infected with rabic virus, Webster and associates¹ successfully immunized mice and dogs against a subsequent injection of active virus. Salk, Lavin, and Francis² compared the antigenic potency of epidemic-influenza virus following irradiation with that of active virus. In high concentrations, irradiated virus was nearly as effective an immunizing antigen as active virus; when lower concentrations were tested, a hundredfold loss in immunizing capacity was found to have occurred during irradiation. Ultraviolet light has been applied to the virus of equine encephalomyelitis, Eastern strain (E.E.E.), by Sharp and associates;³ they studied the molecular stability of ultraviolet-treated virus. The preparation of an immunizing antigen produced by irradiation of E.E.E. virus with ultraviolet light is reported here.

Chick embryos 7-days-old were inoculated with 0.1 cc 10^{-8} suspension of E.E.E. virus-infected embryo in 0.85% saline solution. Embryos removed from the egg 18-20 hours after inoculation were rinsed in saline solution, ground in a mortar and made to a 10% suspension in saline or Tyrode's solution. This suspension was centrifuged at 2000 rpm for 10 minutes; the centrifugation was repeated with the supernate; the supernate then obtained was spun in a Swedish angle-centrifuge at 4000 rpm for 45 minutes. About 30 cc of the final supernate were transferred to a quartz test-tube, with an internal diameter of 2.1 cm, which was placed in the center of a quartz-mercury resonance lamp in the form of a spiral* with an internal diameter of 9 cm. The spiral, 15 cm in height, consists of 7 coils. The tube containing viral suspen-

¹ Hodes, H. L., Lavin, G. I., and Webster, L. T., *Science*, 1937, **86**, 447; Webster, L. T., and Casals, J., *J. Exp. Med.*, 1941, **73**, 601.

² Salk, J. E., Lavin, G. I., and Francis, T., *J. Exp. Med.*, 1940, **72**, 729.

³ Sharp, D. G., Taylor, A. R., Beard, D., Finkelstein, H., and Beard, J. W., *Science*, 1940, **92**, 359.

* Manufactured by the Hanovia Chemical Company.

sion was so placed that there was at least one complete coil of the lamp above and below the suspension. About 85% of the energy emitted by this type of lamp is in the line of 2537 Ångström units. The lamp operates at 30 milliamperes and 15,000 volts transformed from 110 volts A.C. The titer of virus before irradiation was regularly 10^8 when injected intracerebrally in albino mice, Rockefeller Institute strain. After beginning of irradiation, samples of virus were removed at 5-minute intervals; the suspension was stirred at each sampling; 0.03 cc of each sample was tested for infectivity by intracerebral injection of each of 4 mice. Inactivation was complete after 15–25 minutes of irradiation.

**Cerebral Resistance to E.E.E. Virus in Mice
after Vaccination with Virus Inactivated
by Means of Ultraviolet Light (UV) or Formalin (F.V.)**

| Virus dilution | Mice: Vaccinated | | Virus dilution | Non-vaccinated |
|----------------|------------------|---------|----------------|----------------|
| | UV | F.V. | | |
| 10^{-2} | ■ ■ ■ | ■ ■ □ | 10^{-7} | ■ ■ ■ ■ |
| 10^{-3} | □ □ □ □ | ■ □ □ □ | 10^{-8} | ■ ■ ■ □ |
| 10^{-4} | ■ ■ □ □ | ■ □ □ □ | 10^{-9} | ■ □ □ □ |

■ = 1 mouse died □ = 1 mouse survived

FIG. 1

The immunizing capacity of E.E.E. virus inactivated by ultraviolet light was compared with that of formalin-inactivated virus prepared from the same viral suspension, since formalin-inactivated virus has come to be accepted as a standard immunizing antigen. In Fig. 1 the result of one experiment is charted. A group of 11 mice was vaccinated by means of 3 intraabdominal injections on alternate days of 0.25 cc 10% suspension inactivated by 20 minutes' irradiation. Another group was vaccinated with similar doses of virus inactivated with 0.5% formalin. Non-vaccinated mice served as controls. Two weeks after beginning of vaccination, all mice were injected intracerebrally with a broth-suspension of mouse-brain infected with E.E.E. virus in dilutions indicated in Fig. 1. At least half of each group of vaccinated mice survived 10,000 and 100,000 cerebral units. There was no difference between the two vaccinated groups in the degree of immunity induced. It should be added that the antigenicity of such irradiated E.E.E. virus was found to

diminish rapidly with further irradiation, an effect already noted for rabic virus.¹

In summary, a non-infective immunizing antigen has been produced by ultraviolet irradiation of the virus of Eastern equine encephalomyelitis.⁴

⁴ Drs. Casals and Palacios find that E.E.E. virus inactivated by ultraviolet light serves as a highly specific complement-fixing antigen against anti-E.E.E. serum. Their results will be published.

AN INQUIRY INTO THE STRUCTURAL CONDITIONS AFFECTING FLUID TRANSPORT IN THE INTERSTITIAL TISSUE OF THE SKIN

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The formation of lymph is known to be greatly influenced by hyperemia, edema, muscular movement, injury, or venous obstruction (1-5), but the accompanying changes in the tissues are little understood. Is there an increased pressure upon the extravascular fluid under these conditions, and does this drive it into the lymphatics? Is there a change in the resistance of the tissues to the interstitial passage of fluid from blood to lymph? Knowledge of these matters is essential for an understanding of the processes of lymph formation and fluid exchange.

It seems reasonable to suppose that a study of the resistance of the tissues to the passage of fluid under various conditions may throw some light upon the problem presented. In the present work we have studied the resistance offered by normal and edematous cutaneous tissues to the interstitial passage of fluid. In a following paper we will discuss certain changes in this resistance which are associated with various physiological and pathological states as furthermore, certain changes in the pressure exerted upon edema fluid in edematous cutaneous tissue.

Previous Work.—In 1932 Meyer and Holland (6, 7) attempted to find whether fluids forced through cutaneous tissues move as if in capillary spaces or whether they pass between the formed elements like water filtering through fine sand. The authors pointed out that the ratio of the rate of flow of fluid from a cannula into the tissues should increase in simple proportion to the pressure put upon it if there are tissue spaces like channels, leading fluid away from the cannula (Poiseuille's law). On the other hand, if flow through the tissues is a seepage, like seepage through fine sand, the ratio of rate of flow to pressure should be a quadratic relationship. Meyer and Holland obtained evidence that the ratio of flow to pressure was a direct proportion and concluded that interstitial fluid flowed as if in capillaries.

These findings will be discussed in detail further on. For the present it will suffice to say that the work here to be presented will show that the introduction of fluids into the tissues in the amounts required for the measurements of Meyer and Holland must have pushed apart the formed elements and created artificial interstitial spaces.

Recently in this laboratory methods have been developed (8, 9) by which fluids in microscopic amounts can be brought into contact with the tissues of living skin at atmospheric pressure, or at various known pressures, in such a way that they enter neither the blood vessels nor the lymphatics directly. Under these conditions Locke's solution, at atmospheric pressure, is absorbed into the tissues intermittently (8, 9) and we have been able to measure the rate of its absorption. The technique has afforded an opportunity, as will be explained below, to determine with accuracy the resistance of the tissues to the interstitial movement of various fluids brought into contact with them. Observations have also been made upon the changes in the interstitial movement of Locke's solution and other fluids when brought into contact with the tissues under various positive pressures. The findings throw some light upon the nature of the interstitial spaces and the magnitude of the pressures operating in the tissues during the formation of lymph.

Methods

The technique by which exceedingly small amounts of test fluids have been brought into contact with the tissues has already been described (8, 9). It will suffice to recall here that a gauge 30 needle, carrying fluid from a horizontally placed 0.2 cc. pipette, was introduced into the connective tissue of the skin in such a manner that the fluid brought into contact with the tissues did not pass into the blood vessels or the lymphatics directly (8). The movement of fluid in the pipette, occasioned by the entrance of the former into the tissues, was observed through a microscope and measured by the aid of micrometer eyepieces. As the amount of fluid entering the skin was exceedingly small, it was necessary to prevent all movement of the meniscus in the pipette resulting from expansion or contraction of the fluid following the slightest change in room temperature. Accordingly, the apparatus was submerged in a constant-temperature bath (8). An apparatus was also devised, as already described (8), by which the fluid in the pipette and hence the fluid brought into contact with the tissues could be subjected to various pressures. The detailed description of this device, already given (8), need not be repeated here.

The Effects of Pressure upon the Movement of Locke's Solution through the Tissues of the Skin

In 60 experiments we observed the effects of changes in pressure upon the movement of Locke's solution through the tissues of the skin of living mice. It is to be recalled that Locke's solution is readily absorbed from the tissues (9). The experiments yielded consistent results and need not be detailed individually,

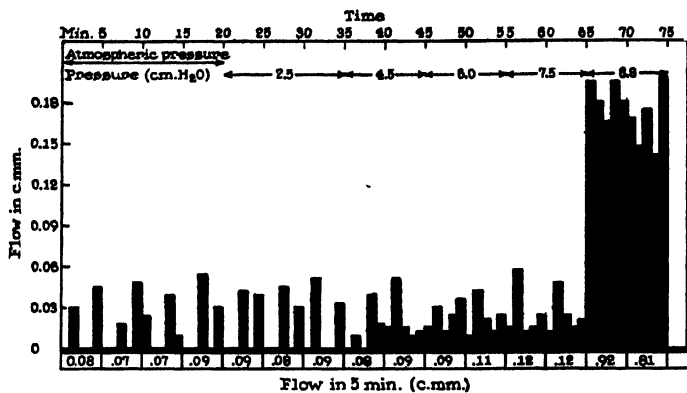
The needle and pipette of the injecting device were filled with Locke's solution at atmospheric pressure and the needle carrying the fluid was introduced into the connective tissue of the skin of the ear or the back of mice anesthetized with nembutal (8, 9). The meniscus of the Locke's solution in the pipette was watched continu-

ously. In all of the experiments it was noted, as in our previous work (9), that the Locke's solution at atmospheric pressure entered the tissues intermittently. When the intermittent entrance of fluid into the skin had been observed for 15 to 20 minutes, that is, long enough to determine its rate and character, the pressure of a column of water 1.0 to 2.5 cm. in height was put upon the fluid in the injecting device, by means already described (8). The subsequent movement into the tissues was observed for 10 to 20 minutes, after which pressure in the pipette was again increased by small amounts for an equal period. Later this procedure was repeated, employing slightly larger or smaller increments of pressure and observing the character and rate of fluid movement all the while until pressures of 20 to 40 cm. of water had been utilized. The findings from two experiments typical of the sixty done are presented in Text-figs. 1 *a* and 2 *a*, in which the data are plotted as described in our previous papers (8, 9).

In all the experiments the fluid continued to move into the skin at approximately the same rate as before when pressures of 1.0 to 2.5 cm. of water were applied; and in about half of them the intermittency of flow remained unchanged as well. An instance of this sort is illustrated by Text-fig. 1 *a*. For the first 20 minutes of the experiment the Locke's solution, at atmospheric pressure, entered the tissues intermittently at quite regular intervals. From the 20th to the 35th minutes, inclusive, as indicated by the numerals between the horizontal arrows near the top of the text-figure, a pressure of 2.5 cm. of water was put upon the fluid in the injection apparatus. Neither the rate nor the character of the flow changed appreciably.

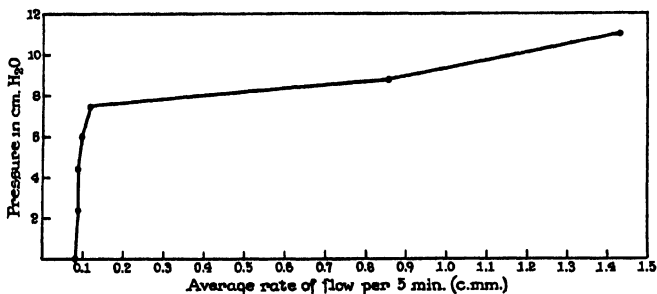
In the other half of the experiments pressures of 1.0 to 2.5 cm. of water produced slight alterations in the intermittency of flow, but the rate of flow did not increase. The periods of inflow lasted a little longer, or recurred at shorter intervals, and the periods of no flow were correspondingly shortened. The data from a typical experiment of this sort appear in Text-fig. 2 *a*. In certain instances, of which this is one, the changes just mentioned became more obvious when increasing pressure was put upon the Locke's solution. (*Vide* Text-fig 2 *a*, from the 30th to the 45th minutes, when a pressure of 3.5 cm. of water was employed in the injection apparatus. It will be seen that the rate of flow into the tissues increased only slightly.)

In practically all of the experiments the flow became continuous when the pressure was raised to approximately 4.5 cm. of water. Text-figs. 1 *a* and 2 *a* both show this fact. The intervals of no fluid movement were replaced by periods of steady flow, separated from one another by periods of slightly greater inflow. It was as if a continuous passage through the tissues had been superimposed upon the ordinary intermittent inflow. In about half the experiments, as *e.g.* in that charted in Text-fig. 1 *a*, there was no change in the rate of entrance of Locke's solution into the tissues, while in the remainder, as typified in Text-fig. 2 *a*, there was an insignificant increase. In all of the experiments further increases of pressure up to 7.5 cm. of water produced hardly any further change, the intake of Locke's solution increasing but little and in many instances (none of which is shown in the text-figures) not at all.



TEXT-FIG. 1 a. The interstitial movement of Locke's solution into the skin of a living mouse at various pressures. The narrow black columns represent the amount of flow of the solution into the skin during each minute of the experiment. A heavy base line has been drawn to indicate that the observations were continuous. The method of plotting the text-figures has been described and discussed in preceding papers (8, 9). In this and all the similar charts, the pressure put upon the introduced fluid is indicated, in centimeters of water, by the numerals placed between the horizontal arrows near the tops of the text-figures.

It will be seen that at atmospheric pressure spontaneous intermittent inflow occurred. Neither the manner nor the rate of inflow was appreciably influenced when pressures of 2.5 and 4.5 cm. of water were put upon the fluid. Only a slight increase in flow was noted when the pressure was 4.5 cm. of water. A pressure of 6.0 caused little further change, and a pressure of 7.5 but little more. At a pressure of 8.8 cm. of water a great and sudden increase of flow occurred, as though the tissues had been forcibly pushed apart.



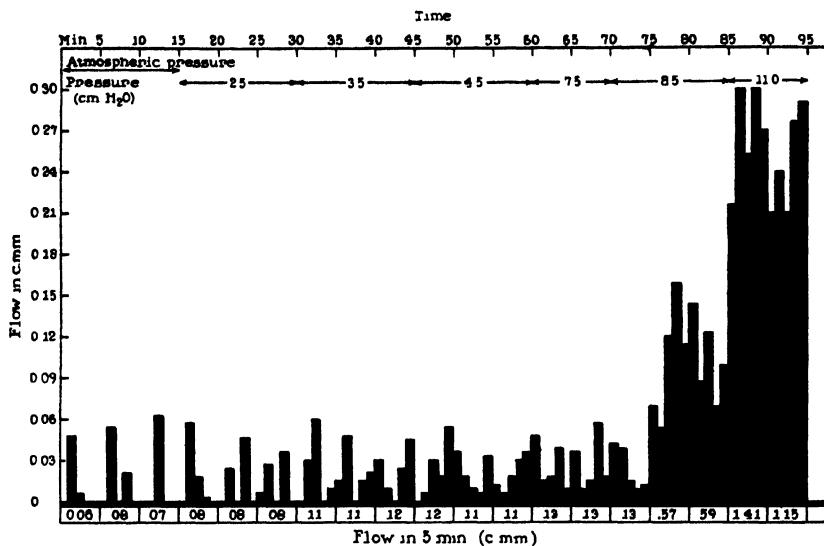
TEXT-FIG. 1 b. The data from the same experiment plotted to show the average rate of entrance of Locke's solution into the skin. It will be seen that inflow increased but little over that occurring at atmospheric pressure when pressures ranging from 1.5 to 7.5 cm. of water were employed. Flow was greatly increased by a pressure of 8.8 cm. of water, and proportionately increased at higher pressures.

Very different were the findings in every one of the experiments when higher pressures were used. The changes then occurring are well illustrated by the typical experiments of Text-figs. 1 *a* and 2 *a*. In both cases, when the pressure on the introduced fluid was increased from 7.5 to 8.8 and 8.5 cm. of water, respectively, there suddenly occurred a great increase in the rate of flow into the tissues, more than quadrupling the previous flow. The great increases in flow followed upon increases in pressure only 1.0 and 1.3 cm. of water higher, respectively, than the pressure previously employed. Apparently at some pressure between 7.5 and 8.5 to 8.8 cm. of water there was a sudden change in the resistance of the tissues to the flow of fluid through them. When this "breaking point," as we shall term it, had been reached, the inflow abruptly became so great that the slight differences produced by the original intermittency were no longer significant, although they still seemed to occur. When in these experiments and in others to be described and discussed below, still greater pressures, up to 20 or 40 cm. of water, were put upon the fluid introduced into the tissues, each further increase in pressure yielded proportionately greater inflow. The data on this point have not been included in the charts.

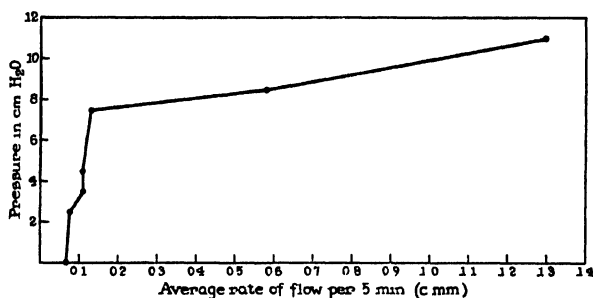
The "breaking point" appeared in every experiment. In a few of them it appeared at pressures as low as 4.5 cm. of water, in a few others not until pressures as high as 12.5 or 14.0 cm. had been brought to bear. In more than 80 per cent of the experiments (49 trials) the "breaking point" appeared at pressures varying from 6.5 to 10.0 cm. of water, and it averaged 8.5 cm. for them all.

The changes in the rate of passage of Locke's solution into the tissues as affected by the various pressures are strikingly shown when the average rate of inflow per 5 minutes at each pressure is plotted. In Text-figs. 1 *b* and 2 *b* this is done for the two typical experiments already considered. A sharp elbow in each curve marks the "breaking point." Additional data not plotted in Text-figs. 1 *a* and 2 *a* have been included, which show that after the "breaking point" had been reached each small increase in pressure put upon the Locke's solution produced some increase in its flow through the skin. More will be said of this phenomenon below. In Text-fig. 3 we have plotted the findings from four other experiments, selecting instances to typify "breaking points" that were low, average, and high, respectively.

Text-fig. 3, line *A*, shows the flow occurring at different pressures in an instance in which the "breaking point" was reached between 4.2 and 4.8 cm. of water. In this experiment Locke's solution at atmospheric pressure entered the tissues at an average rate of 0.12 c.mm. per 5 minutes. At pressures of 2.2, 3.0, and 4.2 cm. of water, flow was only 0.13, 0.14, and 0.14 c.mm. per 5 minutes, respectively. At a pressure of 4.8 cm. of water a sudden increase in flow occurred, which became proportionately greater with increasing pressures.



TEXT-FIG. 2 a. Data from another experiment of the sort plotted in Text-fig. 1 a. In this instance pressures of 2.5 to 4.5 cm. of water did not importantly influence the rate of flow but produced slight alterations in the intermittency of the flow. The periods of inflow endured a little longer or they took place at shorter intervals than they had at atmospheric pressure.

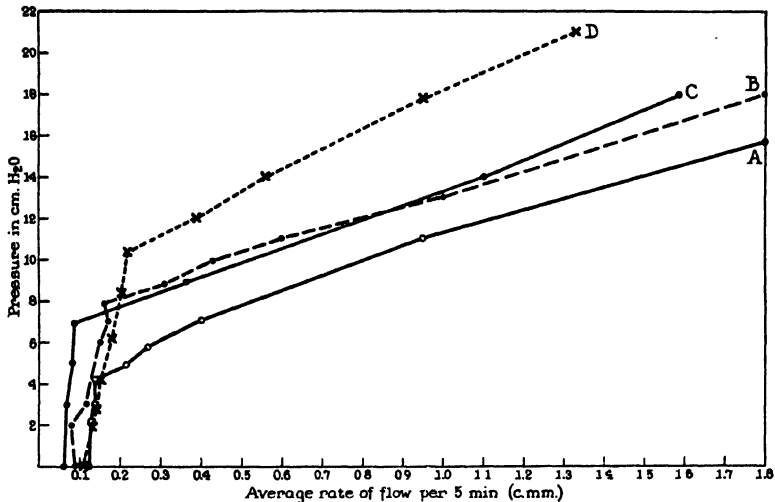


TEXT-FIG. 2 b. Data from the same experiment plotted as in Text-fig. 1 b.

Lines B, C, and D show similar data in three other typical instances. In two of them the "breaking points" lay between 7.0 and 9.0 cm., the average level; while in the third it was not reached until a pressure of 12 cm. of water was exerted.

On comparing these four lines a significant point will be noted. In each experiment measurable flow occurred spontaneously at atmospheric pressure.

Upon the application of low pressures, from 2.0 cm. of water upward until the "breaking point" was reached, there occurred very little increase in the rate of flow, sometimes none. After the "breaking point" had been reached, each addition of pressure produced such an increase in flow that there was a linear relationship between the two, as though the latter were taking place through small channels. This interpretation will be discussed more fully below.



TEXT-FIG. 3. The data from four typical experiments, plotted as in Text-figs. 1 b and 2 b, to illustrate further the effect of changes in pressure upon the rate of entrance of Locke's solution into the skin. Instances of high, low, and average "breaking point" (see text) have been selected. There was a linear relationship between pressure and flow after the "breaking point" had been exceeded.

The Resistance of the Tissues to the Entrance of a Relatively Unabsorbable Edema-Producing Fluid

In the work just reported, such minute amounts of fluid were introduced into the skin, even under pressure, that one could not be certain how much of the increased flow from the apparatus was due to a greater movement of fluid through the tissues and how much resulted from absorption by the blood vessels. The next experiments bore upon this point. They were carried out with a fluid similar in viscosity to Locke's solution but one which calls forth edema, augmenting the fluid bulk within the tissues.

To accomplish our end we have utilized a finding made previously in this laboratory and reported in an earlier paper (9). It was found that the addition to Locke's

solution of $\frac{1}{2}$ per cent of a vital dye, pontamine sky blue, yielded a mixture which failed to enter the cutaneous connective tissue when brought into contact with it at atmospheric pressure. On the contrary, when the mixture was in contact with the skin it called forth an accumulation of fluid in the tissues and after a few minutes this accumulation began to force its way into the injection apparatus, so that the fluid already in the pipette was moved backwards.

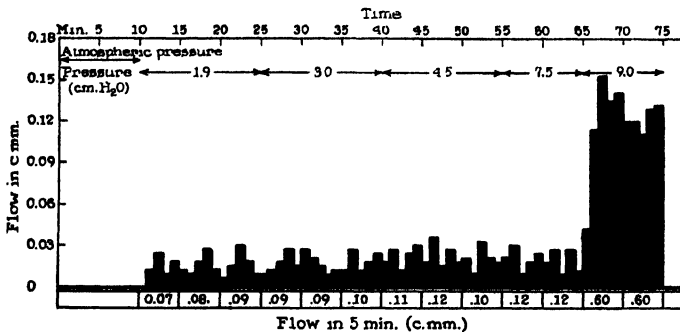
In many other previous experiments (9) the mixture of dye and Locke's solution was brought into contact with the tissues at atmospheric pressure and immediately thereafter forced into the skin at low pressures, that is to say, before fluid had accumulated in the tissues in sufficient quantity to reverse the flow in the injection apparatus or to give rise to an edema visible under the microscope. Under these circumstances the dye-Locke's solution entered the tissues continuously, showing none of the intermittent movement that appears when plain Locke's solution is employed in the same manner. As the phenomenon has been described and the data fully charted in two preceding papers (8, 9), it need not be detailed further.

In the present work, in 40 experiments, the dye-Locke's solution at atmospheric pressure was brought into contact in the usual way with the connective tissue of the skin of the ears or thighs of anesthetized mice. The fluid did not enter except in two instances, which were discarded. In each of the remaining experiments, after it had been ascertained that the dye-Locke's solution failed to enter the tissues during a period of 10 to 15 minutes, the pressure was increased in the injecting device for equal periods of time and by small increments, as in the preceding experiments. When pressures varying from 1.5 to 3.5 cm. of water were applied, the dye-Locke's solution entered the tissues at rates varying between 0.03 and 0.09 c.mm. per 5 minutes, that is to say, at a rate resembling that of the spontaneous entrance of plain Locke's solution at atmospheric pressure. The inflow was not intermittent as in the experiments made with plain Locke's solution, Text-figs. 1 *a* and 2 *a*, but on the contrary was continuous and irregular, as in the experiments of our earlier work just mentioned (8, 9).

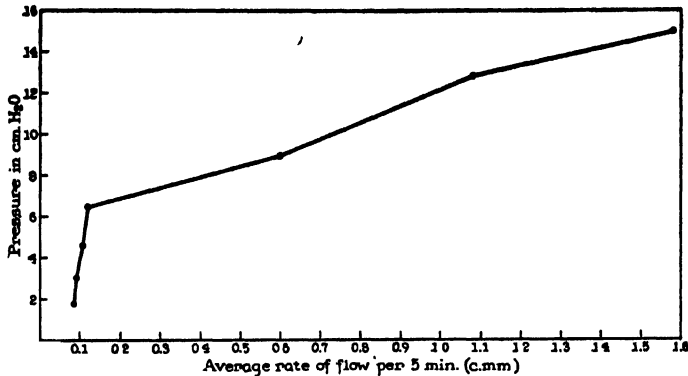
From these findings it is apparent that there were factors making against the entrance of the dye-Locke's solution and that these were overcome by pressures of 1.5 to 3.5 cm. of water. As in the trials with plain Locke's solution, the rate of inflow was increased but little, sometimes not at all, by pressures ranging from 1.5 to 7.5 or 8.0 cm. of water. But with a greater increase a "breaking point" was reached at which a slight further addition in pressure brought about a great and sudden increase in flow. In rare instances the sudden inflow of fluid took place at a pressure of 5.0 cm. of water, and in occasional trials it did not occur until pressures as high as 11.0 to 12.5 cm. of water were exerted, but on the average it occurred at a pressure of 8.5 cm.

Text-figs. 4 *a* and 4 *b* show the results of a typical experiment. It will be seen that the findings differ from those of the previous charts in the ways just

mentioned above. There was no inflow of the dye-Locke's solution mixture at atmospheric pressure. When forced into the tissues at pressures of 1.9,



TEXT-FIG. 4 a. The passage into the tissue of an edema-forming fluid, dye-Locke's solution. The data, plotted as in preceding figures, show that no fluid entered the tissue at atmospheric pressure, and that it entered continuously at pressures of 1.9 and 3.0 cm. of water. There was little increase of flow when the pressure was increased to 4.5 and 7.5 cm. of water, but finally, a sudden, great inflow at a pressure of 9.0 cm. of water. Evidently the "breaking point" had been exceeded.

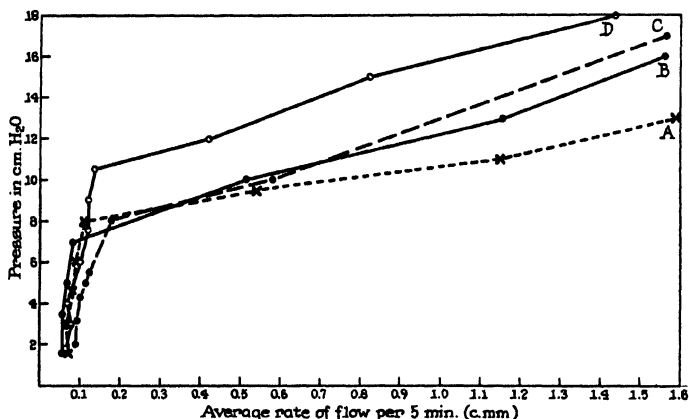


TEXT-FIG. 4 b. The data of the experiment from which Text-fig. 4 a was drawn, plotted to show, as in Text-figs. 1 b, 2 b, and 3, the changes in the average rate of entrance of the dye-Locke's solution at various pressures.

3.0, 4.5, and 7.5 cm. of water, the flow was not intermittent but continuous although irregular, showing that the resistance of the tissues to the entrance of the fluid was overcome. A pressure of 9.0 cm. of water produced a fivefold increase in the rate of the entrance of fluid. The findings at pressures higher than 9.0 cm. of water have been omitted from the figure. In Text-fig. 4 b we

have charted the data from this same experiment to show the average rate of inflow for each 5 minute period at the various pressures employed, up to 15 cm. of water.

Text-fig. 5 shows the data from four typical experiments of the sort plotted in Text-fig. 4 *b*. In some of the four, as e.g. in this experiment, there was little change in either the rate or the manner of fluid entrance into the skin at pressures below the "breaking point"; in some the rate of flow increased slightly; while in others an increase in pressure resulted in a decrease in the observed flow of fluid into the skin. It is clear from this that the observed



TEXT-FIG. 5. Changes in the average rate of entrance of dye-Locke's solution at various pressures. The data are plotted as in the preceding text-figure. Each increase in pressure after the "breaking point" had been exceeded brought about a corresponding increase of interstitial flow, as evidenced by the straightness of the lines.

differences in flow at different pressures below the "breaking point" often fell within the margin of error of the method. In all the experiments, after the "breaking point" had been reached each increase in pressure brought about a corresponding increase in the flow of the dye-Locke's solution through the tissues. As result, the later slant of the curves is approximately straight.

Resistance to the Entrance of Serum

Experiments like those just described were repeated, using homologous serum, a relatively unabsorbable and viscous fluid. It is well known that serum injected interstitially is absorbed slowly.

Fresh, sterile mouse serum obtained from pooled specimens of mouse blood taken with aseptic precautions was brought into contact with the tissues of the skin of the

ears or thighs of 34 anesthetized mice. In about half the number, as in previously reported work (9), the serum, at atmospheric pressure, entered the tissues at the extremely slow rate of 0.01 to 0.02 c.mm. per 5 minutes, about a third the rate of Locke's solution under similar conditions. In the other tests no fluid entered. Only these instances were employed in the present work. After 15 to 20 minutes had elapsed with no entrance of fluid, the pressure within the apparatus was raised by stages, as in the preceding experiments, until at last flow took place at a rate like that of the spontaneous flow of plain Locke's solution into the skin at atmospheric pressure. It was found that pressures of between 1.5 and 4.5 cm. of water sufficed to bring this about. The flow was continuous and slightly irregular like that occurring in the experiments made with dye-Locke's solution as just described.

As in the experiments with the dye-Locke's mixture or plain Locke's solution, further slight increases in pressure produced little increase in flow until a "breaking point" was arrived at. Then there occurred a sudden and greatly increased flow.

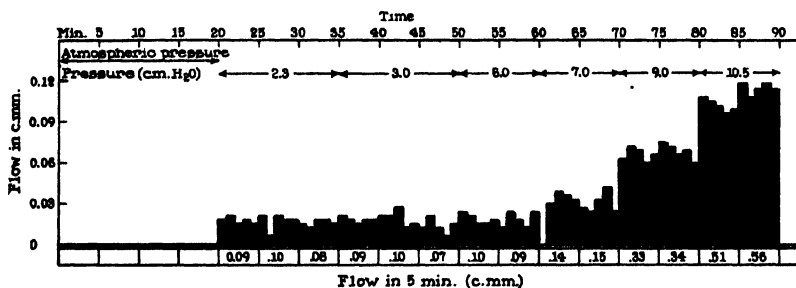
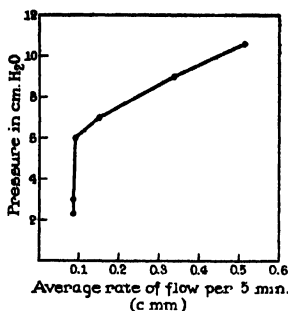
The findings of a typical experiment appear in Text-figs. 6 *a* and 6 *b*. They show that the "breaking point," as indicated by the commencement of sudden, rapid inflow into the skin, occurred at approximately the same pressure as in the tests made with Locke's solution or with the dye-Locke's mixture. In the experiments in which serum was employed the increase of inflow after the "breaking point" had been reached was not so great or so abrupt as in the trials employing the other solutions.

The Nature of the "Breaking Point"

Findings in the Skin of Dead Animals.—In the experiments so far described the "breaking point" was reached at the same pressure whatever the nature of the fluid employed. One may infer therefore that it was determined by the mechanics of the situation, by the bulk of the fluid introduced into the tissues whereby some structural change or separation of the formed elements was effectuated. To exclude the possibility that a circulatory change might have been responsible for the sudden entrance of fluid into the tissues, experiments like those described were repeated on animals that had been killed with ether 1 to 5 hours previously.

Plain Locke's solution, dye-Locke's solution, and homologous serum were used, respectively. We have already shown in a preceding paper (9) that Locke's solution and the dye-Locke's mixture at atmospheric pressure fail to enter the skin of killed mice. Homologous serum also has failed to enter in about half of the trials made, and it passed into the skin very slowly in the remainder. When pressure was brought to bear on these fluids, all three entered into the tissues continuously at pressures of 1.5 to 5.0 cm. of water and there was no sign of the intermittency of flow that appears when Locke's solution is brought into contact with living skin at atmospheric pressure or forced into it at low pressures.

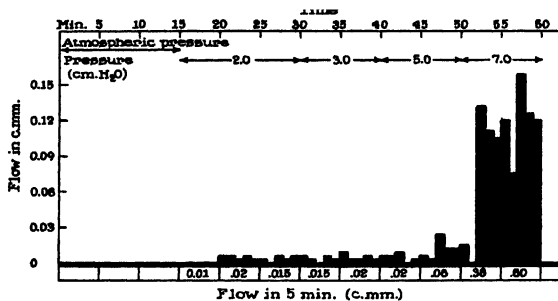
Text-figs. 7 and 8 (*a* and *b*) show the results in two typical experiments out of 26 made on killed mice. In the experiment illustrated by Text-fig. 7, plain Locke's solution was employed; in that represented by Text-fig. 8, a mixture of Locke's solution with $\frac{1}{2}$ per cent of dye. In both instances the test fluids failed to enter the tissues when brought into contact with the skin at atmospheric pressure. Subjected to pressures of 2.0 to 5.0 cm. of water, the fluids entered the tissues in a continuous

TEXT-FIG. 6 *a*TEXT-FIG. 6 *b*

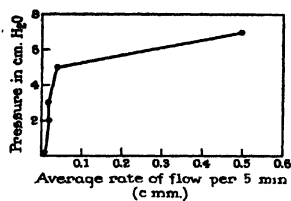
TEXT-FIGS. 6 *a* and *b*. The rate of entrance of a relatively unabsorbable fluid homologous serum, at various pressures.

manner, and there was almost no sign of the intermittent flow which takes place when Locke's solution is forced by similar pressures into living skin (9). It will be noted that in both experiments a sudden increase in the rate of entrance of the test fluids ("breaking point") appeared when the pressure of the introduced fluid was raised to 7.0 and 9.5 cm. of water, respectively.

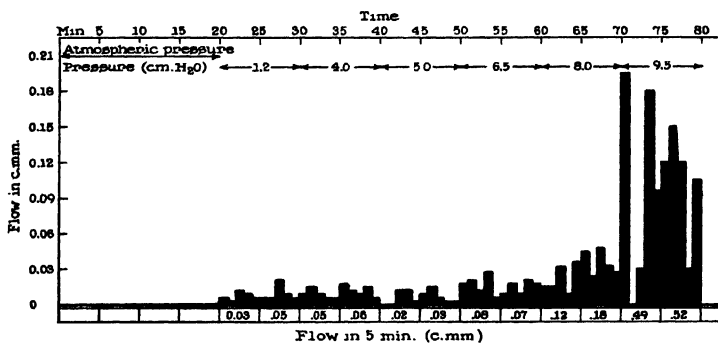
The findings in these two experiments were like those obtained with living animals except for the fact that plain Locke's solution did not enter the skin at atmospheric pressure. The other twenty-four experiments of this group yielded similar results. "Breaking points" appeared at the same pressures



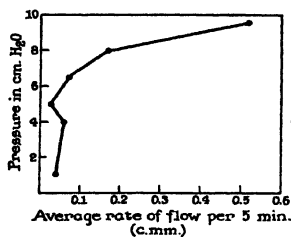
TEXT-FIG. 7 a



TEXT-FIG. 7 b



TEXT-FIG. 8 a



TEXT-FIG. 8 b

TEXT-FIGS. 7 a, b, and 8 a, b. The rate of entrance of Locke's solution (Text-fig. 7) and of edema-forming dye-Locke's solution (Text-fig. 8) at various pressures into the skin of recently killed mice.

as in the experiments on living animals, that is to say, at a pressure of about 8.5 cm. of water on the average. In rare instances "breaking points" appeared at a pressure of 5.0 cm. of water or failed to appear until the pressure was raised to 12.0 cm. of water. The findings showed clearly that the resistance to the entrance of fluid into the skin of recently killed animals is like that offered by the skin of the living. It follows that the circulation has nothing to do with it. The conclusion seems justified that the "breaking point" is due to the giving way of some structural barrier to inflow.

The Entrance of Fluid into Edematous Skin

Edema of the skin much affects the entrance of fluid.

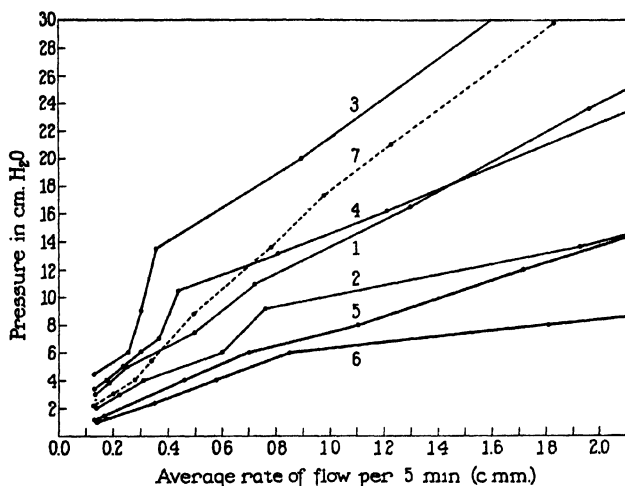
In 39 experiments edema was induced in the skin of the ear by painting it with xylol. This procedure had been found effective in inducing edema in scores of earlier experiments (10-14), as shown by the appearance of the skin under the microscope and by "pitting on pressure" exerted with a small needle. The edema was pronounced in the present experiments and the appearance of the skin proved a sufficient indicator.

In all of the 39 experiments the dye-Locke's solution was employed and observations on the entrance of fluid into the tissues were made as already described. In fourteen cases observations were begun 15 to 20 minutes after painting the ear with xylol, that is to say, while the edema was developing (12). In ten of the remaining 25 experiments tests were made $1\frac{1}{2}$ to 6 hours after painting the ear with xylol, in eight instances 20 to 24 hours later, and in seven instances 4 to 11 days later. In them all the test fluid was brought into contact with the connective tissue of the skin in the usual way and the fluid reservoir was then opened to the atmosphere, to find whether the tissue contained freely movable edema fluid under pressure. When that was the case it flowed back into the pipette. The backflow was always intermittent. It occurred in twelve of fourteen instances in which studies were made within an hour after painting with xylol, that is to say, while edema was developing. It also took place in all of the ten instances investigated $1\frac{1}{2}$ and 6 hours after induction of edema, but only in half of the eight studied 20 to 24 hours after the xylol painting, despite the fact that the ears were still swollen. Still later, 4 to 11 days after edema had been induced, backflow occurred into the injection apparatus in six of the seven trials made. In all seven edema of the skin was still visible at this time.

After it had been ascertained that edema fluid was present, the pressure in the injection apparatus was raised by small increments for varying periods of time and the changes in the rate of flow inwards of the dye-Locke's solution were followed as in the preceding experiments.

In Text-fig. 9 the findings from seven typical experiments are given, to show the changes in the rate of inflow of the dye-Locke's mixture when introduced into the edematous skin under various pressures. The curves are plotted as in Text-figs. 3 and 5. We have omitted from them the initial backflow into the apparatus which occurred in most of the experiments when the pipette was opened to the atmosphere. It is noteworthy that in most of the instances

there was no well defined elbow in the curve, at best an ill defined one, or often indeed none at all, like that which is indicative of the existence of a "breaking point" when fluid is forced into normal skin (*vide* Text-figs. 3 and 5 and the other figures plotted in the same way). The instances showing evidence of an elbow were usually those with the least or most recent edema, as will appear further on.



TEXT-FIG. 9. The rate of entrance of dye-Locke's solution at various pressures into edematous skin. The relationship of pressure to flow is plotted as in the preceding text-figures of the same sort. In contrast with what they show, each small increase in pressure in the present instance was attended by a significant increase in the rate of flow. In some instances there was nevertheless a "breaking point" beyond which fluid entered faster.

Lines 1, 2, 3, and 4 represent the findings in four of the fourteen experiments made an hour or less after painting the ears with xylol, that is to say, during the formation of edema. Lines 1 and 2 represent typical findings from instances which showed much free edema fluid, as judged by the amount of backflow into the apparatus during the preliminary test of conditions. Line 3 is a typical curve plotted from the findings in an instance which showed very little edema fluid, and line 4 is taken from one of the two instances of the fourteen studied during the development of edema, in which there was no backflow into the apparatus although the skin was obviously edematous. It is to be noted that lines 1 and 2 do not mount like lines 3 and 4, that is to say, relatively low pressures had a much greater effect on the entrance of fluid in the experiments from which the first pair of lines was drawn. Further, it is of

interest that lines plotted from experiments in which there was little or no demonstrable edema fluid, lines 3 and 4 respectively, show an elbow, as if the edema had not wholly done away with a "breaking point." These findings are typical of the data from experiments that have been omitted from the figure for the sake of simplicity. In about half of the instances showing much edema fluid the findings were generally like those plotted in line 1, yielding no evidence of a "breaking point," whereas in the others a fairly well defined elbow appeared, as in line 2, in spite of the presence of edema fluid.

The two lowest lines, 5 and 6, in Text-fig. 9 show the findings in two instances typical of the ten experiments in which the tests were begun $1\frac{1}{2}$ to 6 hours after painting the ear with xylol. In every instance backflow occurred at atmospheric pressure, showing that there was free fluid present in the skin. Lines 5 and 6 are drawn from experiments made 5 and 4 hours, respectively, after painting the ear with xylol. In both of these instances there was much backflow into the apparatus at the beginning of the experiment when the dye-Locke's solution was brought into contact with the tissues at atmospheric pressure. The dotted line 7 gives the findings in an experiment made 5 hours after painting the ear with xylol. In this instance there was only a little demonstrable edema fluid and the line slants more sharply upward than lines 5 and 6. It is plain that the test fluid introduced into the skin in this experiment at increasing pressures did not pass into the tissues as readily as in the instances that showed much free edema fluid. Nevertheless there was no evidence of a "breaking point."

The findings from the experiments made 20 to 24 hours or more after painting the ears with xylol are not shown, for when plotted the data yielded lines similar to those numbered 5 and 6.

The findings plotted in Text-fig. 9 have been selected as typifying the changes that occurred, but deviations were frequent. The rate of movement of fluid introduced at a given pressure was not always greatest when the skin contained most fluid as evidenced by the grade of edema, nor was the flow inwards at a given pressure always greater in ears edematous several hours than in those painted only an hour before. Some of the individual differences can be explained no doubt by differences in the pressure under which the edema fluid was held, or by differences in the interstitial pressure, a factor to be discussed in a later paper.

As already noted, in some instances if the edema fluid in the tissues was apparently scant, there was suggestive evidence of a partial "breaking point," as indicated by a suddenly lessened slant of the plotted lines.

On comparing Text-fig. 9 with Text-figs. 1 *b* to 8 *b*, inclusive, it will be seen that the lines in the latter run almost vertically in the early part of the experiments, showing that pressures between 2.0 and 8.0 cm. of water effected no significant increase in the movement of fluid into the skin. Not until the

"breaking point" was reached did a significant increase in flow take place. In Text-fig. 9, on the other hand, the slope of the lines shows that each small increase in pressure above that required to initiate flow into the tissues resulted in a significant increase in the rate of flow. This was true, to a greater or less extent, in all of the 39 experiments on edematous ears. The lines are far from vertical in the first portion of Text-fig. 9 and as the pressure was raised many of them became approximately straight. The significance of this difference will be discussed below.

DISCUSSION

The findings throw light upon the manner of movement of interstitial fluid through connective tissues. The resistance of dermal tissue to the entrance of the test fluids at the rate at which Locke's solution is taken up at atmospheric pressure was negligible. But the skin offered a definite obstacle to the entrance of fluid at a faster rate. Regardless of the fluid employed, the rate of flow into the skin did not increase appreciably as pressure was increased, until a "breaking point" was reached. Since no relationship was found between the rate of fluid entrance into the skin and the pressure employed until this point had been attained, the findings yielded nothing to suggest the presence of preformed spaces or channels through which fluid might stream. On the contrary they indicated that there are no such spaces or channels. But after the "breaking point" had been reached each further increase in pressure led to proportionate increases in the rate of flow of the introduced fluid irrespective of the character of this, with result that a linear relationship developed between the increases in pressure and the flow. This was roughly constant for each animal but differed from individual to individual, as the slope of the lines in the text-figures show.

At pressures above the "breaking point" fluid moved through the tissues as though in small spaces or channels. From the fact that the "breaking point" occurred at the same level regardless of the fluid employed, one can infer that it was determined by the mechanics of the situation, by the bulk of the fluid introduced. The possibility that circulatory changes could account for it has been ruled out by the experiments which showed that it occurred at the same pressure in the skin of living animals and in those killed with ether. Obviously it signified a separation of the formed elements, resulting from the pressure of the introduced fluid. Inevitably such a change must always occur when fluids are injected into tissues by hand, as in clinical medicine, for under these circumstances, as will be shown in later work, the pressure of injection is far higher than that required to exceed the "breaking point."

This abrupt change in the characteristics of fluid movement through the interstitial tissue of the skin makes plain a fact suggested by earlier work (13, 14), that interstitial fluid does not exist normally in tissue spaces large enough to permit it to move freely.

When edema was present in the skin the characteristics of interstitial fluid movement changed much. After flow into the skin had been initiated, by exerting pressure, at the rate at which Locke's solution is taken up by normal skin at atmospheric pressure, each increase of pressure, however slight, led to a corresponding increase in the rate of flow. The latter was more rapid than in normal skin at corresponding pressures, showing that the resistance of the edematous skin to the passage of fluid at these pressures was less than normal. The relationship of this change to the formation of lymph in edematous skin will be discussed in a following paper after data have been presented on the pressure of edema fluid under these circumstances.

In more than half the instances of edema studied no "breaking point" appeared as the pressure was raised and throughout the experiment a linear relationship continued to exist between the pressure employed and the rate of flow. It is known that in edematous skin the formed elements of the tissues are separated slightly by the fluid. One might expect that the entrance of more fluid under these circumstances would take place as if through preformed spaces, and our findings show that this is actually the case.

In certain of the experiments upon edematous skin, a fairly well defined "breaking point" did appear. These were usually instances in which the skin had only recently become edematous (Text-fig. 9). One may infer that under such conditions spaces may not have opened up to the same extent as later and that in consequence the tissue may yield further when pressure is brought to bear.

One further point deserves mention. As already stated, Meyer and Holland (6, 7) concluded from their work that fluids move through normal tissues as though through capillary spaces. The reason for their conclusion is now clear. They forced 0.6 per cent NaCl solution into the subcutaneous tissues at pressures much above the "breaking point" demonstrated in the present work. Indeed they injected the fluid into the tissues at the rate of 30.0 to 40.0 c.mm. a minute. Having ascertained the pressure required to yield that rate of flow, they lowered the pressure until the saline solution entered the tissues at the rate of 10.0 c.mm. a minute. Plotting the rate of flow against the pressure employed, they estimated by extrapolation the pressure at which no flow would theoretically occur. In each experiment the tissues must have been forced apart to begin with by pressures above the "breaking point" and thereafter the rate was measured of flow through the artificial tissue spaces that had been created.

SUMMARY

With the aim of determining the structural conditions which affect fluid movement in the cutaneous connective tissue of mice, various test fluids were brought into contact with it under conditions such that neither blood vessels

nor lymphatics were directly entered. Locke's solution, mouse serum, and a mixture of Locke's solution with a dye which causes edema were all employed. At atmospheric pressure, Locke's solution entered the tissues intermittently. When subjected to very low pressures it continued to enter the skin intermittently and at approximately the same rate. At pressures above 4.5 cm. of water, however, the flow became continuous but it did not increase in rate significantly until pressures of about 8.5 cm. were employed. There was no relationship between the rate of flow and the pressure employed. At a pressure of about 8.5 cm. the resistance of the tissues seemed to give way abruptly as if the formed elements had been separated. This has been termed the "breaking point." After it had been reached each further increase of pressure produced a proportionately greater inflow.

Under the conditions of our experiments, the dye-Locke's solution and also the homologous serum failed to enter the tissues at atmospheric pressure. It was necessary to subject these fluids to pressure to force them into the skin at the same rate at which the Locke's solution entered it spontaneously. Under these circumstances the dye-Locke's solution and the serum entered the skin continuously, not intermittently like the plain Locke's solution. As the pressure was gradually raised, no significant increase of flow into the tissues occurred until a point was reached, on the average 8.5 cm. of water, at which fluid suddenly began to enter very rapidly. This point, the "breaking point" already mentioned, was reached at the same pressure irrespective of the character of the fluid employed, showing that the phenomenon was produced by the fluid bulk. Once it had been attained, further increases in pressure caused proportionately greater inflow of fluid. The circulation had nothing to do with the phenomenon, for it occurred in the skin of dead mice.

The findings indicate that under normal circumstances the movement of fluid in the interstitial tissue does not take place as though in preexisting channels. The experiments confirm previous observations from this laboratory (13, 14) that in normal skin tissue the state of affairs is such that fluid cannot flow freely. However, when fluid is introduced into the skin under pressure spaces are forcibly opened up.

Inflammatory edema in the skin changed the phenomena of fluid entrance into it under pressure. The reason is that there then occurred a separation of the formed elements and the interstitial fluid moved as in preformed channels. Even when very low pressures were employed (3.0 to 7.0 cm. of water), there appeared usually a linear relationship between the pressure and the rate of flow.

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A METHOD TO DETERMINE THE PERIPHERAL ARTERIAL BLOOD PRESSURE IN THE MOUSE

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PLATES 4 AND 5

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A method for the determination of the blood pressure of the mouse without injury to the animal will be briefly described in the present paper, together with certain observations upon the changes of pressure under various physiological conditions. The method has the advantage that it enables the pressure to be taken while injections into a vein of the tail are in progress.

Previous Work.—Recently Bonsmann (1) reported a method to determine the blood pressure in the tails of mice and rats. A specially designed cuff enclosed the tail except for its tip which lay under a photoelectric cell. Blood was driven out of the tail by pressure in the cuff, and when it was allowed to return the resulting change of color in the tip of the tail was registered by the cell and the pressure was read off. More will be said of Bonsmann's findings below. As the method employed the animal's tail, it was not suited to our purposes. Still more recently Diaz and Levy (2) and Williams, Harrison, and Grollman (3) have published excellent methods to determine the blood pressure in the rat, but their methods also involve utilization of the animal's tail.

Griffith (4) has reported a method to measure the blood pressure in the legs of the rat. In his work the blood vessels of the skin of the foot were observed while pressure was exerted on the thigh by means of a cuff. We have modified the method of Griffith, observing the return of blood to the claws of the hind feet of mice.

The Method

The claws of the hind legs of mice were found to be transparent to the focused and cooled beam of a carbon arc light. Under these circumstances, as will be shown below, the entire circulation of the nail bed, including the afferent and efferent vessels and the capillaries, becomes visible under a binocular microscope. One can see with ease the moment at which peripheral circulation ceases in the claw, if pressure is exerted higher up on the leg by a cuff, as in clinical methods for determining blood pressure. One can note too with great accuracy the moment that arterial circulation begins anew when pressure is gradually released. Readings can be made more accurately by

observing the circulation in the claw than by watching the circulation in the skin of the leg, for, as Griffith (4) has pointed out for the rat, there is difficulty in determining whether or not the smaller cutaneous vessels seen under the microscope are arterial or venous.¹

Mice of 16 to 35 gm. body weight were anesthetized by a single intraperitoneal injection of nembutal or luminal. Nembutal was given as a 1 per cent solution, 0.5 cc. per 25 gm. of body weight, luminal as a 2 per cent solution in doses of 0.125 cc. for every 10 gm. of body weight. In the dosages employed, the luminal usually produced a deeper and longer anesthesia, but, as will be seen below, the depth of anesthesia varied greatly from animal to animal at any given time after the injection.

The Pressure Apparatus.—It is difficult to arrange a blood pressure cuff about the upper part of the leg of small mice, for the leg is so shaped that the cuff when inflated tends to slip toward the foot. It was necessary to prevent this in some way. The following means was employed:—

The board on which the anesthetized mouse lay consisted of pieces of cork cemented together. It was hollowed out, as shown in Fig. 1, and two movable strips of celluloid were laid over the hollow. These strips could be shifted to accommodate animals of different sizes. Two smaller celluloid plates, 6 cm. long and 2 cm. in height, were set upright and parallel to each other into other smaller pieces of cork board, as shown in the figure. These celluloid strips stood 1.5 cm. apart. Through each plate of celluloid three holes were bored, 1 cm. in diameter, large enough to allow the anesthetized animal's hind legs and tail to pass through without meeting obstruction. When the animal was placed on its back, as shown in the photograph, and one leg was drawn through the holes, a segment of the thigh 1.5 cm. in length lay between the two celluloid plates and about 0.7 cm. above that portion of cork board shown in the figure at A.

A small rubber blood pressure cuff had been constructed, of such size that when deflated it could be passed about the animal's thigh, just filling the space between the parallel pieces of celluloid and the board at A. A ribbon-like strip of steel, just wide enough to fit between the celluloid plates and bent in a semicircle, was placed over the cuff while the latter was still deflated, as indicated at C in Fig. 1. Through holes bored in both celluloid plates pins, not shown in the figure, were passed horizontally just above the metal strip. When inflation of the cuff was begun, the pins prevented the metal strip from rising. As result, the cuff was held against the leg by the metal strip above, by the cork board below, and by the celluloid plates at each side, and was so retained by these structures that pressure came upon that portion of the leg which lay between the celluloid plates. The latter, in addition, prevented the cuff from slipping up or down the leg. The cuff was connected with a mercury or water manometer in the usual way and with an inflating bulb.

To make visible the circulation in the nail, the rays of a Leitz carbon arc light were cooled by passage through 5 cm. of water and focused, by an adjustable concave mirror, upon the claw to be examined. For simplicity this part of the apparatus has been omitted from the photograph in Fig. 1. The brilliant light transilluminated the

¹ It may be noted in passing that the circulation in the claw of rats, even of young ones, can be seen only with difficulty.

claw and when the room was in darkness the circulation of blood and movement of the individual red cells in the capillaries could be easily seen with the aid of a microscope. In each experiment the light was focused in turn upon each of the five claws and the circulation in each examined. The claw yielding the clearest picture of its blood vessels was selected. The blood vessels were best seen when the rays of light entered on the convex surface of the claw.

The vascularization of two claws is shown in Figs. 2 and 3. To obtain Fig. 2 an anesthetized mouse, 27 gm. in body weight, was slowly injected into a vein with 1 cc. of a solution of Higgins India ink diluted six times with a 0.9 per cent NaCl solution containing 1.3 per cent of gelatin. After this had entered the circulation, the animal was bled from the jugular vein while a second cubic centimeter of the ink solution was injected. A few minutes after the injection the foot was removed and placed in ice water for 15 minutes and then in cold alcohol for half an hour, after which it was partially cleared in methyl salicylate for 24 hours. The vessels, magnified 100 times as seen in the photograph, have approximately the same caliber as in life and appear much as they do during the measurement of blood pressure. In order to avoid dilatation of the vessels it was necessary to make an incomplete injection, and as result the picture does not bring out all the vessels present. Further, in a photograph it is impossible to include all the vessels since they cannot be brought into one plane. The richness of the circulatory bed of the claw is better demonstrated in Fig. 3, which was obtained under the same circumstances as Fig. 2, except for the fact that the injection material consisted of 4 cc. of a mixture of 8 per cent gelatin solution with equal parts of undiluted Higgins India ink. It will be seen on comparison with Fig. 2 that the smaller vessels are much dilated. During life blood flows toward the periphery in the smaller vessels of the plexus at the center of the picture and returns in the two large marginal vessels.

The measurement of blood pressure was carried out in the following manner. The cuff was rapidly inflated to a pressure estimated to be just above the systolic blood pressure, which will be seen below to vary somewhat with the stage of anesthesia and the condition of the animal. If the movement of blood continued in the claw, the pressure in the cuff was raised slowly until it ceased. When cessation of circulation was attained, the pressure in the cuff was lowered by stages of 10 mm. of mercury, allowing it to remain at each level for 2 minutes. When flow first appeared in any of the vessels of the claw, the pressure was recorded and the cuff deflated. After 2 or 3 minutes the cuff was again blown up, this time to a pressure a few millimeters of mercury higher than that just recorded. When all flow in the claw ceased, the pressure was lowered, 2 or 3 mm. of mercury at a time, with a wait of a minute or two at each new pressure, until a slight movement of red cells appeared in one or two of the small vessels in the central portion of the plexus near the base of the claw, the region indicated in Fig. 3 by an arrow. The slight movement usually lasted for only

a few seconds and then ceased, as though it had been caused by a readjustment of fluid in the vessels and not by true blood flow. In most instances no further flow took place and a minute or two later the pressure in the cuff was lowered 2 or 3 mm. of mercury. As result, a pulsating flow of cells, toward the extremity of the toe, usually made its appearance, first in one or two channels, then in most of the vessels of the plexus. In some instances, without any lowering of the pressure, the slight initial movement of cells was followed by the pulsating flow, while in rare cases this failed to appear until the pressure had been lowered several times by 2 or 3 mm. of mercury. As a rule, a few seconds after the pulsating flow of cells began the flow became continuous through all the small channels of the plexus. For a little while, as the pressure was maintained in the cuff, the cells accumulated in the larger collecting vessels at the edge of the plexus, but after half a minute, or slightly more, blood flow usually established itself in these vessels too. If the circulation failed to become complete within one or two minutes the pressure in the cuff was reduced by 2 or 3 mm. of mercury and invariably flow appeared in all the vessels. If the final reduction of pressure was not made, the circulation eventually established itself, but sometimes only after several minutes.

For reasons to be discussed below, we have taken as the systolic blood pressure in the leg that pressure found in the cuff at the moment when the pulsating flow of blood first appeared in the small vessels of the claw and became continuous. As routine, to avoid undue congestion of the foot, pressure in the cuff was released as soon as the systolic pressure had been determined. In all instances two pressure determinations were regularly made in the manner just described and the average of the readings was taken, if agreement was good. When the readings varied by more than 5 mm. of mercury, a third determination was made and the three readings averaged.

Control Experiments

The question arose, were we measuring the true systolic pressure in the large arteries of the thigh or did the apparatus merely obstruct blood flow in the skin and claw? To test this point two series of control experiments were made. In the first, dye was injected into a tail vein while various pressures were maintained in the pressure cuff. In the second series of control experiments blood pressure was determined directly from the carotid artery and compared with simultaneous measurements obtained from the leg by the method just described.

Results of the Injection of Dye into the Circulation during Measurement of the Blood Pressure.—In twelve experiments, after the systolic blood pressure had been measured in the leg as just described, the cuff was inflated to a pressure higher by 2 to 4 mm. of mercury. After ascertaining that flow in the claw had ceased, 0.05 cc. of a 5.4 per cent, isotonic solution of a vital dye, pontamine sky blue, was injected into the tail vein. This dye solution, the preparation of which has been described (5), has been used by us in larger or smaller doses in much previous work (5-8). The injections

employed here colored the animals well except in the portion of the leg and foot below the pressure cuff. After a few minutes, during which color still failed to appear in the occluded foot, the pressure in the sphygmomanometer cuff was lowered by a few millimeters of mercury until the pulsating flow of red cells made its appearance in the minute vessels of the claw.

This procedure invariably was attended by blue coloration of the leg and foot. This had been absent previously, showing that there was no blood flow to the tissues of the foot or lower leg at pressures above the one we accepted as the peripheral systolic pressure.

The second series of control experiments involved simultaneous blood pressure readings in the leg by the method described above, and in the carotid artery by direct cannulation of the latter.

Direct Measurement of the Carotid Blood Pressure of the Mouse.—Mice anesthetized with nembutal or luminal were injected intravenously with 0.1 cc. of a heparin solution, 10 units to the cc. They were placed on the board shown in Fig. 1, covered with light layers of cotton, and kept warm by means of electric lights placed near the body. Half an hour later one carotid artery was exposed and cannulated with a gauge No. 27 hypodermic needle, employing a binocular microscope for its insertion. During this manipulation the artery was occluded with a rubber-tipped bulldog clamp. To prevent all loss of blood during the measurement of blood pressure, the apparatus shown in Fig. 1 was employed. The cannulating needle, previously filled with heparin solution, was connected with a three-way stopcock and this in turn was affixed to a 0.2 cc. Bureau of Standards pipette bent at right angles, as shown in the figure. The pipette was also filled with heparin solution but contained a minute droplet of mercury in the center of the graduated portion. A manometer in circuit with a device by which any desired pressure could be brought upon the contents of the pipette was connected with the latter, as shown in the figure. The device need not be described in detail here as that has already been done in earlier work (9), while furthermore, the principles of its operation are clear from the photograph (Fig. 1).

During the insertion of the needle into the artery and while it was being tied in with very fine silk thread, the stopcock was turned in such a way that the contents of the needle was shut off from that of the pipette. The clamp on the artery was then released, and a pressure of about 100 mm. of mercury was put upon the contents of the pipette. The stopcock was then turned for a second or two to allow communication between the needle and the pipette-manometer system. Since the pressures in the pipette and the carotid artery were usually not equal, the droplet of mercury in the pipette began to move either toward or away from the artery. The stopcock was immediately closed, and the pressure in the pipette-manometer circuit either increased or decreased by raising or lowering the leveling bulb *B*, shown in Fig. 1. Again the stopcock was opened to permit communication between the needle and the pipette and the droplet of mercury allowed to return to its original position. These adjustments were repeated as often as necessary until the pressure in the pipette just balanced that in the carotid artery and the mercury droplet merely pulsated in the pipette but did not flow in either direction.

A Comparison of the Findings Obtained by the Two Methods Just Described.—

In fourteen experiments simultaneous readings of blood pressure in the carotid artery and in the leg were successfully obtained. As a rule, the reading of the manometer attached to the carotid artery was a few millimeters higher than that attached to the cuff when the cells in the minute vessels of the claw first manifested the pulsating movement already described, which later, and without change in cuff pressure, became continuous. Better agreement between the two methods was sometimes found if the pressure in the cuff was read at the moment when the first irregular movement of cells occurred in the smallest vessels of the obstructed claw. But, as already mentioned, the first irregular movement of cells in the small vessels of the claw sometimes ceased and was not resumed again unless the cuff pressure was lowered by a few millimeters of mercury. It was felt that this movement might simply be the expression of readjustment of blood in the vessels following the early release of pressure in the cuff and the phenomenon was not as clear-cut as the appearance of the pulsating flow.

In some experiments many readings were taken by observation of the claw and while doing so the cuff was repeatedly inflated or deflated. In these instances pronounced edema of the foot and lower leg developed. Under these circumstances the first appearance in the capillaries of the pulsating flow which later became continuous occurred at cuff pressures which became progressively lower than the direct carotid blood pressure readings, eventually by as much as 10 to 15 mm. of mercury. The finding will be discussed below. It was evidently advisable to make as few blood pressure determinations as possible in any one experiment and none was attempted if swelling or edema of the foot appeared.

A comparison of the blood pressure findings obtained by the two methods appears in Tables I to III, which will be discussed below. For the present it will suffice to say that a comparison of the figures in the 4th and 5th columns indicates a remarkably good agreement. From this it is clear that the method for determining blood pressure by observation of the circulation in the claw is adequate for most purposes.

Variations in the Blood Pressure of Mice

Under the circumstances of our studies the blood pressure of the mice differed greatly, and the pressure in individual animals also varied much from time to time. Because of the lack of data on the blood pressure in mice, it seems necessary to present a record of the variations we have encountered, in order that others may know of them who wish to employ the method described.

It is generally known that animals under deep anesthesia have a lower blood pressure than those lightly anesthetized, and furthermore, that blood pressure

TABLE I

The Blood Pressure of Mice Anesthetized with Nembutal

| Experi- ment No. | Time after injection of nembutal | Stage of anesthesia | Carotid blood pressure | Cuff pres- sure | Remarks |
|------------------------|-------------------------------------------|------------------------|------------------------------|-----------------------|------------------------------|
| | | | mm. Hg | mm. Hg | |
| 1 | 44 | Very light | 111 | 108 | |
| | 50 | " | 112 | 112 | |
| | 60 | " | 118 | 113 | |
| | 70 | " | 121 | — | |
| 2 | 50 | " | 119 | 114 | |
| | 60 | " | 117 | 114 | |
| | 80 | " | 120 | 115 | |
| | 35 | Deep | 88 | 80 | |
| 3 | 50 | Light | 96 | 92 | |
| | 70 | Very light | 101 | 104 | |
| | | | | | |
| 4 | 90 | Light | 126 | 120 | |
| | 98 | " | 120 | 115 | |
| | 120 | " | 83 | 83 | |
| 5 | 52 | " | 108 | 106 | |
| | 59 | " | 108 | 105 | |
| | 67 | " | 102 | 100 | |
| | 74 | " | 100 | 98 | |
| | 82 | " | 95 | 85 | |
| | 92 | " | 90 | 87 | Edema appearing in the foot |
| | 107 | " | 82 | 72 | Edema pronounced in the foot |
| | 117 | " | 74 | 64 | " |
| | 142 | " | 64 | 53 | " |
| | 147 | " | 46 | 37 | Animal moribund |
| 6 | 55 | Deep | 96 | 94 | |
| | 65 | " | 90 | 87 | |
| | 80 | " | 72 | 68 | |

The blood pressure measurements from the carotid and by way of the pressure cuff on the leg were made simultaneously.

TABLE II

The Blood Pressure of Mice Anesthetized with Nembutal

| Experi- ment No. | Time after injection of nembutal | Stage of anesthesia | Carotid blood pressure | Cuff pressure |
|------------------------|-------------------------------------------|---------------------|------------------------------|------------------|
| | | | mm. Hg | mm. Hg |
| 7 | 70 | Very light | 124 | 124 |
| 8 | 67 | Light | 108 | — |
| 9 | 90 | Very light | 102 | 100 |
| 10 | 50 | " | 102 | 97 |
| 11 | 47 | Moderate | 100 | — |
| 12 | 70 | Light | 90 | — |
| 13 | 60 | Deep | 97 | 96 |
| 14 | 45 | " | 86 | — |
| 15 | 80 | " | 82 | 78 |

TABLE III

The Blood Pressure of Mice Anesthetized with Luminal

| | | | | |
|----|----|------|----|----|
| 16 | 90 | Deep | 96 | 94 |
| 17 | 45 | " | 74 | 68 |
| 18 | 45 | " | 68 | 63 |

The blood pressure measurements from the carotid and by way of the pressure cuff on the leg were made simultaneously.

varies greatly with changes in the physiological state. Our findings in the mouse are in accord with this knowledge (Tables I to III).

The physiological state of the animals varied much from instance to instance. In some animals cannulation of the carotid artery was done rapidly and with ease, in others slowly and with difficulty. The trauma of the operation and manipulation of the needle in the tissues of the neck must have much influenced matters. Further, we desired to measure the blood pressure in animals as lightly anesthetized as possible.

TABLE IV

| Experiment No. | Time after injection of the anesthetic | Blood pressure in the leg "Cuff method" | Comment on depth of anesthesia |
|----------------|----------------------------------------|-----------------------------------------|-------------------------------------------------------------------------------------------------------|
| | <i>hrs.</i> | <i>mm. Hg</i> | |
| 1 | $\frac{1}{8}$ | 76 | No response on pricking tail: deep anesthesia |
| | $\frac{3}{8}$ | 84 | Response on pricking tail: moderate anesthesia |
| | 1 | 105 | Occasional movement of legs: light anesthesia |
| | $1\frac{1}{2}$ | 109 | Frequent movement of legs: very light anesthesia; at 1 hr. 40 min. withdrew leg from cuff and ran off |
| 2 | $\frac{1}{4}$ | 80 | Response to pricking tail: moderate anesthesia |
| | $\frac{1}{2}$ | 82 | " " " " " " |
| | $\frac{3}{4}$ | 98 | " " " " " " |
| | 1 | 104 | Occasional movement of legs: light anesthesia |
| | $1\frac{1}{4}$ | 106 | " " " " " " |
| | $1\frac{1}{2}$ | 114 | Frequent movement of legs: very light anesthesia |
| | $1\frac{3}{4}$ | — | At 1 hr. 44 min. withdrew leg from cuff and ran off |
| 3 | $\frac{1}{8}$ | 72 | No response on pricking tail: deep anesthesia |
| | $\frac{3}{8}$ | 88 | Slight response on pricking tail: moderate anesthesia |
| | $1\frac{1}{4}$ | 98 | Occasional movement of legs: light anesthesia |
| | $1\frac{1}{2}$ | 118 | Frequent movement of legs: very light anesthesia; at 1 hr. 32 min. withdrew leg from cuff and ran off |

Blood pressure readings made by the "cuff method" alone in mice anesthetized with nembutal and subjected to no trauma. As anesthesia became lighter the blood pressure rose.

The amount of nembutal given was just sufficient to maintain anesthesia during the average time required for cannulation of the carotid artery. As the time required to perform the cannulation varied much, the initial blood pressure readings were made in some instances as early as 35 minutes after giving the anesthetic (Experiment 3, Table I), while the animals were still deeply under its influence. In other instances, as in Experiments 4 (Table I) and 9 (Table II), the initial blood pressure readings were not obtained until $1\frac{1}{2}$ hours after giving the nembutal, and the animals were in a state of light narcosis rather than anesthesia. Apart from these differences, the response of some of the animals to the uniform dose of nembutal varied much. Some remained deeply anesthetized for periods of approximately $1\frac{1}{2}$ hours, Experiments 6 (Table I)

TABLE V

| Experiment No. | Time after injection of the anesthetic | Blood pressure in the leg "Cuff method" | Depth of anesthesia | Experiment No. | Time after injection of the anesthetic | Blood pressure in the leg "Cuff method" | Depth of anesthesia |
|----------------|----------------------------------------|-----------------------------------------|---------------------|----------------|----------------------------------------|-----------------------------------------|---------------------|
| | hrs. | mm. Hg | | | hrs. | mm. Hg | |
| 1 | 1½ | 70 | Deep | 10 | 1½ | 91 | Moderate |
| | 1¾ | 84 | Moderate | 11 | 1½ | 87 | " |
| | 2 | 87 | Light | | | | |
| | 2¼ | 91 | " | 12 | 2 | 90 | Light |
| | 2½ | 100 | " | | 2½ | 103 | Very light |
| | 3 | 106 | Very light | 13 | 2¼ | 92 | Light |
| | 3½ | 109 | " " | | | | |
| 2 | 1½ | 62 | Deep | 14 | 1 | 69 | " |
| | 2 | 62 | Moderate | | 1½ | 68 | " |
| | 2½ | 62 | " | | 2 | 76 | " |
| | 3 | 95 | Light | 15 | 1 | 93 | Very light |
| | 3½ | 102 | Very light | | 1¼ | 88 | " " |
| 3 | 1 | 72 | Deep | | 1¾ | 86 | " " |
| | 1¼ | 72 | " | 16 | 2½ | 93 | " " |
| | 1¾ | 82 | Moderate | | 1 | 105 | " " |
| | 2¼ | 82 | Light | | 1½ | 112 | " " |
| | 2½ | 93 | Very light | | 2 | 111 | " " |
| | 3 | 93 | Very light | | 2½ | 114 | " " |
| 4 | 1½ | 69 | Deep | | 3 | 114 | " " |
| | 1¾ | 76 | " | | | | |
| | 2¼ | 87 | Moderate | | | | |
| | 3 | 94 | " | | | | |
| 5 | 1 | 96 | Deep | | | | |
| | 1½ | 90 | " | | | | |
| | 2 | 72 | " | | | | |
| 6 | 1 | 81 | " | | | | |
| | 1½ | 81 | " | | | | |
| | 2 | 92 | " | | | | |
| 7 | 2½ | 60 | " | | | | |
| | 3 | 62 | " | | | | |
| 8 | 1¼ | 78 | " | | | | |
| | 2 | 80 | " | | | | |
| 9 | 1¾ | 71 | " | | | | |

Blood pressure readings made by the "cuff method" alone in mice anesthetized with luminal and subjected to no trauma. The table shows a correlation between the depth of the anesthesia and the level of blood pressure.

and 15 (Table II). The three animals given luminal (Table III) also remained deeply anesthetized.

In all the experiments the depth of anesthesia or narcosis was judged from time to time by the animal's response to a light puff of air into the nostrils or a slight pin prick in the skin of the tail. In the tables the depth of anesthesia at the time of each reading has been recorded as "deep," "moderate," "light," or "very light." By deep anesthesia is meant surgical anesthesia with loss of reflexes, by moderate anesthesia a stage in which the animal responded to the stimuli mentioned above by a momentary twitch. During light anesthesia mice without direct stimulation made occasional spontaneous movements tending to withdraw the leg from the cuff, and when the state of anesthesia was very light these movements became frequent.

In Table I, Experiments 1 to 3 summarize the findings from three of six animals which recovered from the anesthetic sufficiently to withdraw the leg from the pressure cuff a few minutes after the last readings were taken. It is clear from this fact that the anesthesia had almost entirely worn off. In all instances, the lighter the anesthesia the higher were the blood pressure readings. In three other experiments (4 to 6, Table I) the blood pressure fell as time passed. The data of Experiment 5 illustrate the fact mentioned earlier, that repeated estimations of blood pressure by the cuff method sometimes led to the development of a discrepancy between the pressure readings taken from the leg and those obtained from the carotid artery. In this instance the experiment lasted for $2\frac{1}{2}$ hours, and the animal obviously suffered from shock and exposure. Ten measurements of blood pressure in the leg were made, each in duplicate or triplicate. The foot showed visible edema after the fourth determination, as indicated in the table, and with the passage of time the edema increased. Similar findings appeared in other experiments, which need not be included in the table.

Table II gives the data from nine experiments in which single pressure readings only were attempted. It will be seen that there was a rough correlation between the level of the blood pressure and the depth of anesthesia when judged as described earlier. Finally, Table III shows the blood pressure readings that were obtained from three animals anesthetized with luminal. Though all were in the state of deep anesthesia, the first animal $1\frac{1}{2}$ hours after giving the anesthetic showed a higher blood pressure than the others $\frac{3}{4}$ of an hour after receiving luminal.

Tables IV and V show well the variations in blood pressure that have been found in mice which were deeply, moderately, or lightly anesthetized with nembutal or luminal. In these experiments the animals were not operated upon and they lay quietly on a warming pad with one leg in the pressure cuff. In most of the experiments blood pressure determinations were made from time to time while the animals recovered from the anesthetic. The three mice of Table IV recovered sufficiently to withdraw the leg from the pressure

cuff and run off. Inspection of Table V shows that in most of the lightly anesthetized mice the blood pressure was relatively high 1 to 2 hours after injecting the anesthetic (Experiments 12 to 16, inclusive). In the animals deeply anesthetized (Experiments 1 to 9) the initial readings were low in comparison with the later ones.

COMMENT

The data of the tables indicate, as might have been expected, that the blood pressure of mice lightly anesthetized with nembutal or luminal was higher than that of animals deeply anesthetized.

Bonsmann (1), studying the blood pressure in the tail of mice by means of a pressure cuff and photoelectric cell, found a 20 to 45 per cent reduction in the pressure after administration of 30 per cent of the fatal dose of chloral hydrate and morphine. The blood pressure of the mouse as reported by him varied from 70 to 100 mm. of Hg, figures lower than those of the present work in which it varied from 60 to 126 mm. of Hg. We attribute the difference to the accuracy of the present method.

SUMMARY

Advantage has been taken of the relative transparency of the claw of the mouse to devise a method, here described, to measure the blood pressure in the animal's leg. Direct measurements of the systolic blood pressure from the carotid arteries of anesthetized mice have also been made. Simultaneous blood pressure readings by both these methods applied to the same animal showed close agreement.

The systolic pressure ranged from 60 to 126 mm. Hg, according to the conditions.

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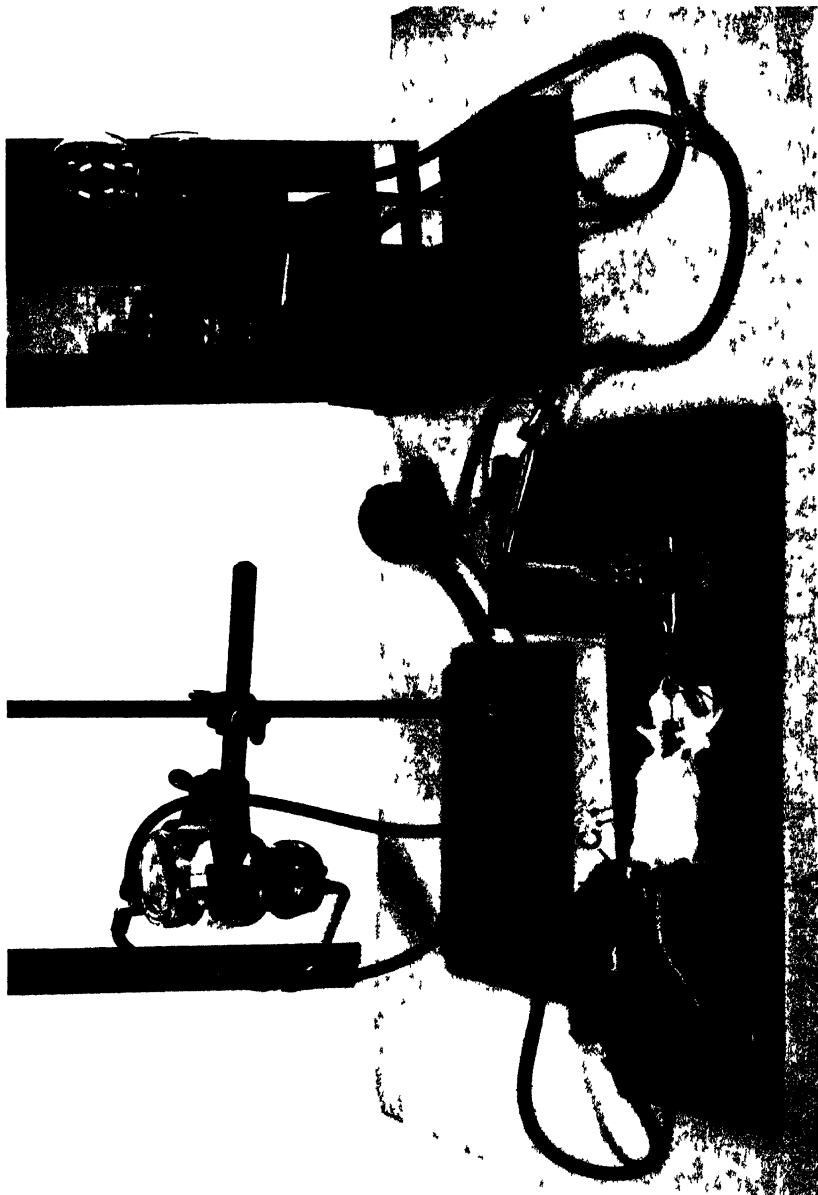
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EXPLANATION OF PLATES

These photographs were made by Mr. Joseph B. Haulenbeck.

PLATE 4

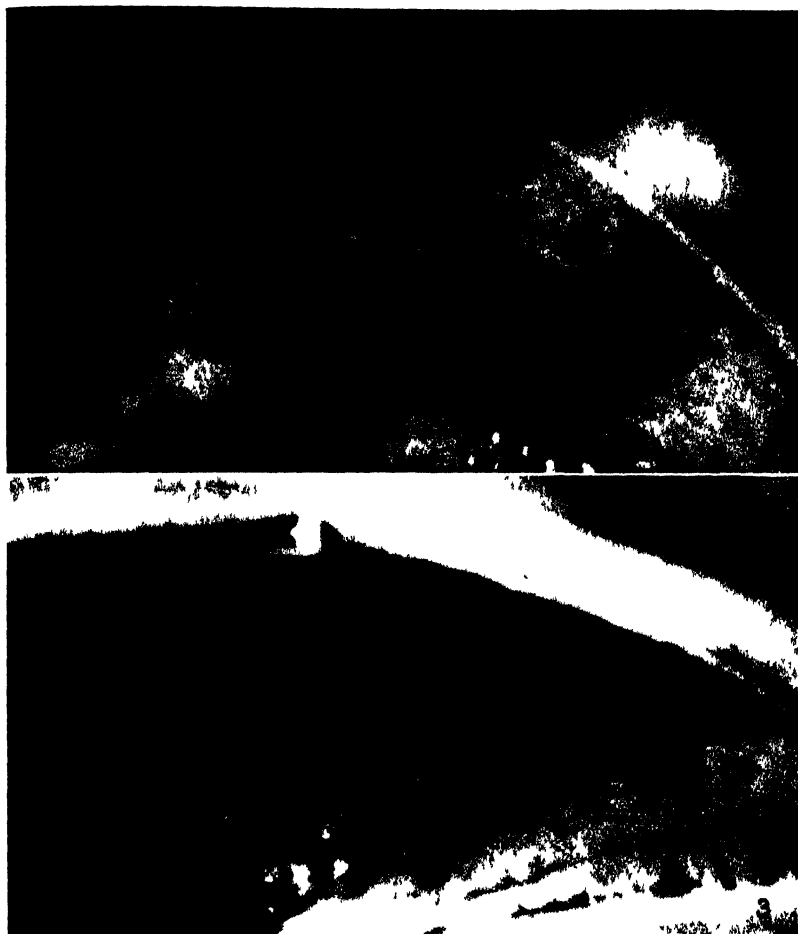
FIG. 1. Apparatus for the determination of blood pressure in the mouse by way of a pressure cuff on the leg and the carotid artery (see text).



(McMaster Peripheral arterial blood pressure in mouse)

PLATE 5

FIGS. 2 and 3. Blood vessels in the claw of the mouse after injection of a gelatin mass containing India ink. $\times 100$.



(McMaster Peripheral arterial blood pressure in mouse)

A NEW GROWTH FACTOR REQUIRED BY CERTAIN HEMOLYTIC STREPTOCOCCI

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Studies on the factors essential for the growth of certain hemolytic streptococci have been in progress for some time. Woolley and Hutchings (1) were able to cultivate on a medium containing only chemically pure substances organisms that belonged to groups B and D of Lancefield's classification. Similar results were subsequently reported by other workers (2). Woolley and Hutchings further demonstrated that organisms of Lancefield's groups A, C, and E would not multiply on similar media. The present work will demonstrate that certain organisms of Lancefield's group A hemolytic streptococci require a factor, hitherto undescribed, for their multiplication on a chemically defined medium. The purification of this substance, as well as many of its properties, will be described below.

Materials and Methods

Organisms Used.—For purposes of routine assay, hemolytic streptococcus strain X 40, originating from the American Type Culture Collection, was employed. Stock cultures were maintained on blood broth.

Basal Medium and Assay Technique.—The basal medium contained the substances listed in Table I. Aliquots of solutions to be tested were added at the expense of the water so that the final volume in every case was 10 cc. The pH was adjusted to 7.4. All constituents except sodium thioglycollate and glutamine were mixed and sterilized in an autoclave; these two were added as a sterile solution just prior to inoculation.

Each tube was inoculated with 1 drop (0.05 cc.) of an inoculum prepared in the following manner. The cells from an 8 hour culture of the streptococcus in Todd-Hewitt broth (4) were collected by centrifugation and resuspended in an equal volume of sterile phosphate buffer. After the tubes had been incubated for 40 hours at 37°C., the turbidity of each tube was quantitatively determined as previously described (1). In order to check the results thus obtained, the acid produced in each tube was titrated with N·0.1 sodium hydroxide to the brom thymol blue end-point. In addition, plate counts of the number of organisms were occasionally made. No growth or acid production occurred in those tubes containing the basal medium. Maximal growth accompanied by maximal acid production occurred in tubes that had been supplemented with 1 mg. per cc. of the standard liver preparation (fraction A) described below. Representative data are shown in Table II. Plate counts also indicated that good growth had occurred in those tubes which became acid and appeared turbid. A "maximal unit"

(m.u.) was defined as the quantity of material that must be added per cc. of medium in order to produce maximal growth and acidity. The m.u. value of each fraction examined was determined by comparison of the response elicited by the addition of various quantities of the fractions. In general, a unit based on maximal response is not as precise as one based on half maximal response. However, in the case of the group A streptococci the maximal response has proven easier to determine than half maximum. In each series of assay tubes several dilutions of a standard liver extract were included to serve as reference points of activity.

Sources of the Growth Factor.—Several liver extracts were assayed for relative potencies. The alcohol-soluble portion of aqueous liver extract was found to be ineffective.

TABLE I
Basal Medium

| | |
|---------------------------------------|----------------|
| Acid hydrolyzed casein . . | 20 mg. |
| Tryptophane | 0 5 " |
| Glucose | 20 " |
| K ₂ HPO ₄ | 40 " |
| NaCl | 20 " |
| MgSO ₄ ·7 H ₂ O | 0 8 " |
| FeSO ₄ ·7 H ₂ O | 40 gamma |
| MnCl ₂ | 12 " |
| Thiamin | 5 " |
| Riboflavin | 10 " |
| Vitamin B ₆ hydrochloride | 10 " |
| Sodium pantothenate | 10 " |
| Adenine | 50 " |
| Nicotinic acid | 50 " |
| Choline chloride | 50 " |
| Inositol | 200 " |
| Uracil | 50 " |
| Pimelic acid | 50 " |
| Biotin concentrate (3) | 0 5 " |
| Sodium thioglycollate | 1 mg |
| Glutamine | 50 gamma |
| Water | to make 10 cc. |

The most convenient source was that fraction of aqueous liver extract which was insoluble in 70 per cent alcohol (1 m.u. in 1 mg.). This fraction will be subsequently designated as "fraction A".¹ Yeast extract (Difco) and rice bran extract (vitab) (1 m.u. in 4 mg. and 2 mg. respectively) were also good sources of the active substance. The fact that a deficiency of a known amino acid was not involved was shown by increasing the casein hydrolysate to 1 per cent of the medium; when this was done no growth occurred, unless an active concentrate was also included.

Preliminary Concentration.—It was found that when fraction A was dissolved in a minimum of water and made strongly alkaline with barium hydroxide, the precipitate

¹We wish to thank Dr. David Klein of the Wilson Laboratories for generous gifts of this material.

after decomposition with sulfuric acid contained the active agent. Further purification could be achieved by treating the resulting solution with lead acetate. When from each fraction lead was removed with H_2S , it was found that the activity resided in the filtrate fraction. The active substance was further purified by precipitation with acetone.

Dialyzability of the Active Substance.—At this point in the investigation it was found that the active substance in fraction A was not readily dialyzable, but that certain more purified concentrates contained the active compound in dialyzable form. This finding necessitated a reinvestigation of the many techniques of fractionation which had been used, for it was realized that the dialyzable and the non-dialyzable substances differed in their physical and chemical properties. For example, the non-dialyzable compound was precipitated from aqueous solution by barium hydroxide, whereas the dialyzable substance under similar conditions was not precipitated. The activity residing in fraction A was not rendered dialyzable by standing in N sodium hydroxide or N mineral acid or by heating. The best method for conversion of the non-dialyzable form to its

TABLE II

Responses of Hemolytic Streptococcus Strain X 40 to Graded Amounts of a Liver Fraction*

| Addition of fraction A to basal medium | Colorimeter reading | 0.1 N acid |
|-------------------------------------------|---------------------|-----------------------|
| <i>mg. per cc.</i> | | <i>cc. per 10 cc.</i> |
| None | 100 | 0 |
| 0.1 | 92 | 0.15 |
| 0.5 | 89 | 1.0 |
| 1.0 | 78 | 1.7 |
| 2.0 | 78 | 1.7 |

* American Type Culture Collection.

dialyzable component was the following. 100 gm. of finely powdered fraction A were stirred for 1 hour with 1 liter of alcohol and 20 cc. of concentrated HCl . The insoluble matter was filtered off and washed with alcohol, and the solvent was removed from the filtrate under reduced pressure. The filtrate contained all of the active agent (1 m.u. in 500 gamma) and in a dialyzable form.

Purification Technique.—A typical run will be described to indicate the procedure that has given the most active concentrates.

100 gm. of fraction A were dissolved in 200 cc. of water and dialyzed in a cellophane tube (Visking) for 18 hours against running water. The non-dialyzable portion was treated with saturated lead acetate until no more precipitate formed. Lead was removed from the resulting filtrate with H_2S , and the filtrate from the PbS was concentrated under reduced pressure to about 200 cc. This solution was made alkaline to phenolphthalein with a saturated methanol solution of barium hydroxide. Care was exercised not to use too large an excess of barium for, as will be shown later, the active substance was tenaciously adsorbed by barium sulfate. The precipitate of barium salts was removed by filtration and decomposed by trituration with excess sulfuric acid. The filtrate from the barium sulfate was then treated with saturated lead acetate until no more precipitate formed. The resulting filtrate was freed of lead with H_2S , the lead sulfide was removed by filtration, and the filtrate was concentrated under reduced

pressure to dryness. Various runs have yielded 300 to 800 mg. of this concentrate per 100 gm. of fraction A. Most of the preparations thus obtained contained 1 m.u. in 10 gamma. However, certain preparations have been almost inactive, and this failure has been traced to adsorption by barium sulfate. The active substance and practically all of the solids of concentrates prepared in this manner were readily dialyzable. Thus, when 180 mg. were dialyzed against 8 changes of distilled water, only 5 mg. did not dialyze, and all of the activity resided in the dialyzable portion.

Adsorption of the Growth Factor.—The active substance in the non-dialyzable portion of fraction A was adsorbed on norit only with difficulty. A fractionation procedure designed to exploit this fact has not yielded concentrates more active than those prepared by other means; but a brief description may be advantageous in that it will illustrate certain properties of the growth factor. When the non-dialyzable portion of 100 gm. of fraction A was diluted to 2 liters and adjusted to pH 1 with HCl, the activity was not invalidated by adsorption with 40 gm. of norit. The filtrate from this adsorption was then stirred with 150 gm. of norit and the resulting filtrate was found to contain only 10 per cent of the original potency. The norit was stirred with dilute aqueous ammonia and filtered. The filtrate was inactive. Activity could then be regained from the norit by 3 elutions with 70 per cent alcohol containing 5 per cent concentrated aqueous ammonia. The latter eluate contained approximately 7 gm. of solids and had about 50 per cent of the original potency. Elution could also be accomplished by stirring with alcoholic HCl.

It has not been possible to adsorb the active material from any concentrate with Fuller's earth (Lloyd's reagent), which statement holds true in respect to both the dialyzable and the non-dialyzable forms.

Barium sulfate adsorbed the active compound tenaciously. For example, when the non-dialyzable portion of 100 gm. of fraction A was treated with a solution of 50 gm. of barium hydroxide and then enough sulfuric acid was added to remove all the barium, the filtrate from the barium sulfate was inactive. Attempts to elute the growth factor with alcoholic ammonia or alcoholic HCl have not been successful.

Miscellaneous Properties of the Growth Factor

It may be of use to other workers in the field to describe some of the properties of the active substance that have been observed, without, however, giving minute details of the experiments demonstrating these properties. Most of the properties have not as yet been exploited to bring about marked concentration of the active substance, but many of them have diagnostic value in differentiating this new growth factor from other growth factors that have previously been described. While the dialyzable form was soluble in alcoholic HCl, it was precipitated from alcohol solution when neutralized. Both the dialyzable and the non-dialyzable forms were not destroyed by nitrous acid or by hot acid or alkali. Even concentrated HCl at 100° did not readily destroy the substance. Both forms were precipitated from aqueous solution by phosphotungstic acid, but much loss

of activity was encountered when this reagent was used. The dialyzable form was not precipitated from alcoholic or aqueous solution by mercury salts. Butyl alcohol did not readily extract the substance from aqueous solution. The growth factor was not soluble in acetone, chloroform, or ethyl acetate. Water seemed to be the only solvent that was effective at all pH values. The non-dialyzable form was not precipitated when its aqueous solution was half saturated with ammonium sulfate, but it was precipitated from saturated ammonium sulfate solution; it was not precipitated from saturated magnesium sulfate.

Relation to Other Growth Factors

It will be observed that the basal medium used contained those substances available in pure form which have been shown to act as growth factors for microorganisms. It is not possible to establish definitely a relationship to factors not yet available in pure form. However, the properties of the growth factor differ in many respects from those described for other unidentified growth substances. Thus, the factor for yeast described by Alexander and SubbaRow (5) was soluble in many organic solvents. The factor described by Snell and Peterson (6) for their lactic acid bacteria was readily adsorbed on norit and lead sulfide. In addition, through the kindness of Dr. Snell, it has been possible to test a sample of his active picrate, which was found to be inactive for the organism used in the present experiments. The factors studied by Moller (7) have not been described in detail and hence a relationship cannot be discussed. This statement is true also of several other extracts which have been shown to increase the growth of certain microorganisms.

Pappenheimer and Hottle (8) and McIlwain (2) have reported growth of certain group A hemolytic streptococci on media of known composition. It will be noted that our basal medium contained all the substances used by Pappenheimer and Hottle, except that casein hydrolysate, rather than gelatin hydrolysate, was used. The substances described by McIlwain have proved inactive in our assay procedure.

It is possible that the active concentrates contain more than one essential growth factor. Many fractionation procedures which result in a poor yield of activity could be explained on this basis. It is more probable, however, that the poor yields are attributable to destruction of the active substance during the procedure, for in every case it has been a standard practice to assay the fractions in combination as well as singly. In no case has evidence for a multiple nature of the growth factor been found.

Effect on the Growth of Other Strains and Species

Members of Lancefield's group A hemolytic streptococci other than strain X 40 have been tested in an attempt to learn whether the new growth factor is essential for this group of microorganisms as a whole. Strains S 43, C 203, and 594 were observed not to grow in the basal medium, but to develop satisfactorily in the basal medium plus the most active concentrate. An indication that other species of bacteria require the growth factor was found when it was observed that pneumococcus D 39 R behaved in a manner similar to the streptococci. The rate of growth of yeast in the medium used by Eastcott (9) was markedly increased by additions of the active concentrates.

SUMMARY

A new factor essential for the growth of hemolytic streptococci of Lancefield's group A has been demonstrated. It has not been possible to identify this factor with any other known growth factor. The active substance occurred in liver as a water-soluble, alcohol-insoluble, non-dialyzable material. It could be rendered dialyzable by treatment in strong alcohol with HCl, but not by many less drastic procedures.

The most active concentrates prepared gave a maximal effect when approximately 10 gamma per cc. were added.

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RELATIONSHIP OF PANTOTHENIC ACID AND INOSITOL TO ALOPECIA IN MICE

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It has recently been shown in two laboratories^{1, 2} that mice become hairless over large areas of the body when they are maintained on a purified diet. It has also been shown^{3, 4} that inositol is a curative agent for the alopecia. With the isolation and identification of the anti-alopecia factor, it became of interest to study pantothenic acid deficiency in the presence of all other known essentials and also to determine if there were other unknown vitamins required by mice. It was observed that even in the presence of inositol, but in the absence of pantothenic acid, alopecia developed. The present report deals with alopecia which results from a lack of pantothenic acid and with that which results from a lack of inositol.

A highly purified basal diet which contained no unknown water-soluble constituents was prepared as previously described.⁵ Pantothenic acid in the form of *d,l* sodium pantothenate⁶ and inositol were added in the amounts desired for various experiments. Since only one optical isomer of pantothenic acid is biologically active, the levels of racemic acid quoted throughout this paper must be divided by two in order to obtain the quantity of active material which the animals received. General care of the mice was similar to that previously described.¹

For the study of pantothenic acid deficiency the basal ration was supplemented with 100 mg of inositol per 100 g of ration. For the positive controls, it was further supplemented with 5 mg of pantothenic acid per 100 g. The first sign of pantothenic acid deficiency was retarded growth. After about 3 weeks on the experimental diet, the deficient animals became hyperirritable. A few days later they displayed difficulty in controlling their hind quarters. They seemed to be seized by periodic spasms of pain, for at intervals very violent and rapid movement would take place and the animals would squeal occasionally, as if in pain. The hind quarters sometimes exhibited convulsive,

¹ Woolley, D. W., *J. Biol. Chem.*, 1940, **136**, 113.

² Norris, E. R., and Hauschildt, J., *Science*, 1940, **92**, 316.

³ Woolley, D. W., *Science*, 1940, **92**, 384.

⁴ Woolley, D. W., *J. Biol. Chem.*, in press.

⁵ Woolley, D. W., *Proc. Soc. Exp. Biol. and Med.*, 1940, **43**, 352.

⁶ Woolley, D. W., *J. Am. Chem. Soc.*, 1940, **62**, 2251.

circular movements. After a few days the violent movements became subdued and a more or less complete paralysis of the hind legs ensued. In addition, the eyes of many of the animals were affected and in some cases they were unable to open them. A few days following the appearance of these symptoms, alopecia began to develop on the ventral surface of the body. The hair was lost first just behind the fore limbs on the chest. The denuded areas spread slowly over the entire ventral surface and then up the sides, and finally across the back. Some animals became completely naked except for the head. All of these symptoms were frequently not observed in the same animal. For example, the nervous and muscular manifestations were not invariably followed by alopecia, and alopecia sometimes developed without the appearance of the other symptoms. Occasionally animals died during the severe early manifestations and hence did not survive long enough to lose their hair.

When pantothenic acid was administered to animals at any stage of these conditions, a slow return to normality resulted. The violent movements could be eliminated within 1 or 2 days following the administration of 5 mg of pantothenic acid per 100 g of ration. The effect on alopecia was manifest much more slowly and it was usually necessary to continue treatment for about 3 weeks before restoration of hair began. Increasing the dosage of pantothenic acid up to 1 mg per day did not reduce this period. Eventually, however, the animals assumed a normal appearance.

The symptoms preceding alopecia suggested those observed in pigs⁷ and the syndrome of running fits in dogs.⁸ It will be of interest to test the effect of pantothenic acid in these conditions.

The amount of pantothenic acid which the mouse requires cannot be stated with accuracy. Some indication of the minimum level was found in a series of experiments in which graded doses were given. Definite symptoms of pantothenic acid deficiency (effects on hind quarters, ventral alopecia) were noted with 500 γ per 100 g of ration. A few cases were observed following administration of 1 mg per 100 g. With 2 mg or 5 mg levels, no symptoms were observed and growth was satisfactory.

In the presence of adequate pantothenic acid and in the absence of inositol, alopecia resulted. For these studies the basal ration was supplemented with 5 mg of pantothenic acid per 100 g. After mice had been fed this ration for about 6 weeks, many of them began to lose their hair. The first bare spots were usually seen on each thigh, well up towards the spine. The hairless areas spread rapidly up the back and over the sides. The ventral surface was not affected. Usually the last areas to become affected in pantothenic acid

⁷ Chick, H., Macrae, T. F., Martin, A. J. P., and Martin, C. J., *Biochem. J.*, 1938, **32**, 2207.

⁸ Arnold, A., and Elvehjem, C. A., *J. Am. Vet. Med. Assn.*, 1939, **95**, 303.

deficiency were the first to show a lack of inositol, and vice versa. The difference in affected areas was usually most strikingly apparent in the early stages of the disease.

TABLE I
Incidence of Alopecia on Various Purified Rations

| Addition to 100 g of basal | Total No of animals | No. of cases of alopecia |
|------------------------------------------------------------------|---------------------|--------------------------|
| 100 mg inositol | 9 | 6 |
| 5 mg <i>d,l</i> pantothenate | 39 | 9 |
| 5 mg <i>d,l</i> pantothenate and 100 mg of inositol | 12 | 0 |
| 5 mg <i>d,l</i> pantothenate following a 2-week depletion period | 35 | 19 |

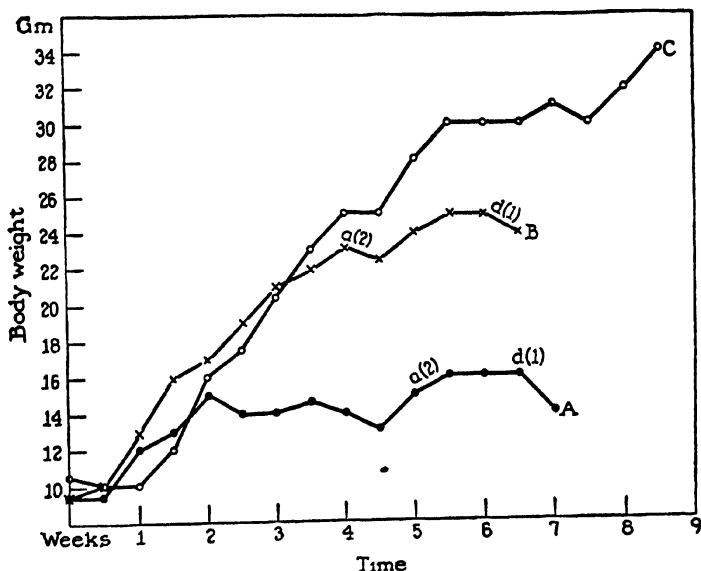


FIG. 1. Growth responses to pantothenic acid, inositol, and pantothenic acid and inositol. Curve A represents the average response of 3 mice receiving inositol; Curve B represents the average response of 5 mice receiving pantothenic acid; Curve C represents the average response of 3 mice receiving both inositol and pantothenic acid. a = alopecia; d = dead; numbers in parentheses represent the number of animals affected.

Administration of inositol to such animals resulted in regeneration of the growth of hair, usually about 10 days after therapy had been started. Soon the animals presented a normal appearance. It was observed that when such

hairless animals were continued without inositol many of them regained their hair; only a very few lost weight and eventually died. For example, in a group of 13 hairless mice, 5 regained their hair within 3 weeks after the onset of the disease, one died, and 7 remained hairless throughout a period of 6 weeks. Some of the animals which had presented spontaneous cure did not exhibit a normal appearance but rather appeared unkempt and somewhat greasy. Such animals could be restored rapidly to a normal appearance by the administration of inositol.

Animals which received both inositol and pantothenic acid grew more rapidly than did those which received only pantothenic acid. With large doses (5 mg. per 100 g of ration) of pantothenic acid the increment of growth produced by inositol was not large, but repeated trials have demonstrated that it was actual. For example, in 3 trials in which a total of 40 animals were employed, the average weights after 7 weeks with no inositol were 27 g, 33 g, and 24 g, whereas with inositol the weights were 26 g, 39 g, and 31 g. The average starting weights were the same.

It was evident that both pantothenic acid and inositol influenced the growth of hair in mice. From the description given by Norris and Hauschildt,² it appears that their animals were suffering from a multiple deficiency. The effect on growth of pantothenic acid deficiency was much more pronounced than was that of inositol deficiency (Fig. 1). Deaths were much more common in the absence of pantothenic acid than in the absence of inositol. The fact that hairless animals receiving pantothenic acid but no inositol in many cases eventually regained their hair raises the question as to what should be called the "anti-alopecia factor." In the presence of inositol, no alopecia developed whereas in its absence hair was lost. Whether or not the effect of inositol was merely to increase the food consumption of the animals cannot be decided because of the impossibility of determining accurately the food consumption of mice. It seems, however, that the inositol effect was not a mere reflection of pantothenic acid effect because of the difference in the early picture of alopecia in the two deficiencies.

The fact that mice may be raised satisfactorily and rapidly to maturity on a highly purified diet is noteworthy. The only constituents of the ration about which there can be uncertainty as to chemical composition are the oils and the casein; all the other constituents were pure compounds. It will be of interest to determine whether the oils and casein can be replaced by pure fat-soluble vitamins and pure amino acids. Whether or not natural foodstuffs contain materials which increase slightly the rate of growth obtained with the purified diet has not been determined.

IDENTIFICATION OF THE MOUSE ANTIALOPECIA FACTOR

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Recently a new dietary essential required by the mouse has been described (1). When this substance was absent from the diet, young mice soon ceased to grow and became completely bald over large areas of the body. Preliminary concentration of the curative substance which was present in liver has been described. Norris and Hauschildt (2) simultaneously and independently described a similar syndrome in mice which were fed a highly purified diet. In the present communication the identification of the curative material will be described. A preliminary statement of our results has appeared recently (3).

EXPERIMENTAL

Assay Technique—In the early part of this investigation the assay procedure previously described (1) was followed exactly. In the last stages, especially after the crystallization of the active substance, the following modifications were introduced. First, the administration of yeast extract to the depleted animals (as with Diet Y) was discontinued and a purely synthetic mixture of water-soluble vitamins (as in Diet S) was employed throughout the test period. In addition, some of the latest experiments were done with 5 mg. of *dl*-sodium pantothenate per 100 gm. of ration in place of the 1 mg. level used previously. The quantity of pantothenic acid had a decided influence on the occurrence and course of alopecia and, when sufficiently high levels were fed, the disease frequently did not result even in the absence of the present antialopecia factor. However, since the relationship between pantothenic acid and the antialopecia factor requires more study,

the details of our investigation of this relationship will be communicated subsequently.

The omission of yeast extract had one noteworthy result. The restoration of hair did not occur as rapidly in animals on a purely synthetic diet as in those fed the yeast extract ration. Yeast extract could not be included from the beginning of the experiments, for, when this was done, no alopecia resulted. For example, two groups of six mice each were fed the purified ration plus 2 per cent of yeast extract from the beginning of the experiment and no cases of alopecia were observed. This fact suggested that the yeast extract was not devoid of the antialopecia factor.

Preliminary Concentration—A statement of the steps employed in bringing about concentration of the active substance will be made in order to illustrate the course of the reasoning which led to the use of phytin. Actual details of the isolation of the active principle will be described below after the effect of phytin has been noted. The source of the vitamin in every case has been the fraction of aqueous liver extract which was insoluble in 70 per cent alcohol; this was the same fraction as previously used. It was designated Fraction A.¹ This material was dissolved in water, dialyzed, and the non-dialyzable portion treated with norit. The active norit filtrate was made alkaline with barium hydroxide and alcohol was added in order to precipitate the active compound. The precipitate was freed of barium and the active substance was rendered dialyzable by heating it with sodium hydroxide. Concentrates prepared in this manner gave the Scherer test for inositol.

Effect of Phytin—The properties of the concentrates suggested that the active substance might possibly be some phosphoric acid ester of inositol. Studies on the distribution of the vitamin in natural products had revealed that cereal grains were relatively rich sources. Thus, for example, 2 per cent of oats was sufficient to bring about slow cure of alopecia. For these reasons it was thought justifiable to test the action of phytin. It was thought at the time that the active substance was a lower ester of inositol but that phytin might possess activity. When 100 mg. of phytin per 100 gm. of ration were fed, hair was restored and resumption of growth occurred.

¹ We wish to thank Dr. David Klein of The Wilson Laboratories for gifts of this material.

Isolation of Antialopecia Factor—While the assays of phytin were in progress, a crystalline material was obtained from our best concentrate by precipitation with lead acetate and ammonia followed by purification with norit and crystallization from alcohol. The crystals melted at 214–216° and contained 39.8 per cent carbon. When fed at a level of 100 mg. per 100 gm. of ration, they caused restoration of hair. Subsequent tests with authentic inositol showed that this material also possessed activity.

Many procedures have been tested for the isolation of inositol from liver Fraction A and the one found most satisfactory will be described. 100 gm. of Fraction A were dissolved in water and dialyzed for 18 hours. The non-dialyzable portion was evaporated to 200 cc. and refluxed with 400 cc. of concentrated HCl for 6 hours. The solution was then concentrated under reduced pressure to a syrup, made alkaline with barium hydroxide, and treated with sufficient alcohol to give a final concentration of 75 per cent. The precipitate was filtered off, washed with alcohol, and decomposed by suspending it in 65 per cent alcohol and passing in carbon dioxide. The filtrate from the barium carbonate was concentrated under reduced pressure to about 200 cc. and treated with saturated lead acetate until no more precipitate formed. The precipitate was removed and the resulting filtrate was treated with 100 cc. of saturated lead acetate. Enough ammonia was added to cause complete precipitation. The precipitate was filtered off and washed and then decomposed with a slight excess of sulfuric acid. Lead sulfate was removed and the resulting filtrate was again made alkaline with barium hydroxide dissolved in methyl alcohol and enough ethanol was added to give a final concentration of alcohols of 70 per cent. After the mixture had stood overnight, the precipitate was filtered off, washed, and then decomposed by suspending it in 70 per cent alcohol and passing in carbon dioxide. The barium carbonate was filtered off; the filtrate was concentrated to a small volume under reduced pressure, acidified with sulfuric acid, and filtered through norit. Alcohol was added to the filtrate until crystallization occurred. The crystals were recrystallized from water by the addition of alcohol. 42 mg. of material were obtained which melted at 218°. Inositol in the same bath melted at 218°.

$C_6H_{12}O_6$. Calculated, C 40.0, H 6.7; found, C 40.2, H 6.7

10 mg. of the crystals were heated with 20 mg. of sodium acetate and 20 cc. of acetic anhydride. The excess anhydride was removed under reduced pressure, the sodium acetate was removed by extraction with water, and the product was recrystallized twice from dilute pyridine; m.p. 212–213°. Inositol hexaacetate in the same bath melted at 213°.

$C_{18}H_{34}O_{13}$. Calculated, C 50.0, H 5.5; found, C 49.5, H 5.2

Effect of Inositol—Simultaneously with the assay of the inositol isolated from liver, authentic inositol was tested. 100 mg. per

TABLE I

Some Effects of Inositol and Derivatives on Nutritional Alopecia of the Mouse

| Diet | Supplement | Level | No. of animals | No. of recoveries in 18 days | Average gain in weight during test period |
|------|-----------------------------------|-------------------------------|----------------|------------------------------|-------------------------------------------|
| | | <i>mg. per 100 gm. ration</i> | | | <i>gm. per day</i> |
| Y | None | | 4 | 1 | 0.2 |
| " | Phytin | 100 | 2 | 2 | 0.7 |
| S | " | 100 | 1 | 1* | 0.4 |
| " | Crystals from liver | 100 | 1 | 1 | |
| Y | Inositol | 100 | 4 | 4 | 0.7 |
| S | " | 100 | 1 | 1 | 0.1 |
| Y | " | 10 | 1 | 1 | 0.5 |
| " | Phosphorylated inositol | 100 | 2 | 2 | 0.4 |
| S | Inositol purified through acetate | 100 | 2 | 2 | 0.5 |

* Recovery not apparent until after 4 weeks on phytin.

100 gm. of ration were sufficient to bring about restoration of hair and resumption of growth. The inositol (1 mole) was then phosphorylated by heating with pyridine and phosphorus oxychloride (7 moles) and, when this material was purified and assayed, it was found to be as active as the original inositol. Lower levels of inositol have been tried and on the yeast extract ration cures have been obtained with as little as 10 mg. per 100 gm. of ration (Table I).

Since inositol is isolated from natural sources and since relatively large amounts must be fed, it is difficult to prove that a small

amount of impurity is not the active material. In addition, slight change of activity with recrystallization cannot be detected, since no quantitative procedure for the assay of the antialopecia factor is available. It is only possible to observe qualitatively whether or not a substance is active. Recrystallization of inositol from dilute alcohol did not destroy its potency. Furthermore, careful purification of inositol hexaacetate by recrystallization from pyridine and then from alcohol, when followed by hydrolysis and further recrystallization of the free alcohol, did not destroy the activity. For these reasons it is believed that inositol or its phosphoric acid ester is the antialopecia factor.

Since only a small amount of inositol could be isolated from the liver fraction and since the amount obtained was not sufficient to account for the observed potency of the liver, a recovery experiment was performed. 100 mg. of inositol were added to the non-dialyzable portion of 100 gm. of Fraction A and the procedure described above was repeated. 56 mg. of inositol were obtained. It was thus evident that only a small fraction of the inositol present was isolated by our procedure.

DISCUSSION

The experiments related above demonstrate that the growth of hair of mice is markedly influenced by inositol or its esters. They further demonstrate that a combined form of inositol occurs in liver. Combined inositol, especially in heart muscle, has been postulated by Winter (4) and by Rosenberger (5) based on amounts isolated before and after autolysis or treatment with alkali. The present work demonstrates that an alcohol-insoluble, water-soluble, non-dialyzable substance occurs in liver which yields inositol upon acid or alkaline hydrolysis. That no free inositol was present in the non-dialyzable concentrates was shown by failure to isolate crystals when acid or alkali treatment was omitted from the procedure.

The relationship of inositol to the growth of hair in the mouse suggests the use of this substance in other species which manifest deficiencies involving the hair. Whether or not inositol is the additional anti-gray hair factor postulated by Dimick and Lepp (6) and by Williams (7) has not been determined.

SUMMARY

The alopecia which developed in young mice raised on a highly purified diet was cured by addition of inositol or of phytin to the ration. Inositol has been isolated and identified in liver concentrates which cure the same type of alopecia.

Observations on combined inositol in liver have been made.

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MANGANESE AND THE GROWTH OF LACTIC ACID BACTERIA

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During analyses of natural products for pantothenic acid by the method of Pennington, Snell, and Williams,¹ it was observed that certain products produced much more rapid growth of the test organism than did optimal quantities of pantothenic acid. These observations seemed to indicate that the basal medium was deficient in something other than pantothenic acid and it was deemed advisable to discover what this growth factor might be. A basal medium composed of 1 per cent glucose, 0.5 per cent peptone, 0.2 per cent K_2HPO_4 , 0.5 per cent sodium acetate, 0.1 per cent yeast extract, and 0.5 per cent hydrolyzed casein and 0.2 γ per cc. of pantothenic acid was prepared. Substances to be assayed were added in appropriate amounts. The media were sterilized in an autoclave (15 pounds, 15 minutes) and inoculated with a diluted broth culture of *Lactobacillus casei* (approximately 14 million cells per tube). Incubation was carried out at 37° for 16 hours and growth was then estimated both by turbidity determinations and by titration of the lactic acid produced.

The best source of the growth factor was malt sprouts or an aqueous extract of malt sprouts. The factor was removed from solution by such simple reagents as alkali or H_2S . It was then found that the ash of malt sprouts was active and that the activity was destroyed by H_2S . Trials of several metallic salts showed that manganese was the effective material. Salts of Cu, Pb, As, Sb, Sn, Hg, Bi, Cd, Tl, Fe, Zn, W, and Mo (0.01 to 100 γ) were without effect or were inhibitory.

Ziatarov and Kalcheva² observed that manganese stimulated *Streptococcus lactis*. Our observations extend these conclusions

¹ Pennington, D., Snell, E. E., and Williams, R. J., *J. Biol. Chem.*, **135**, 213 (1940).

² Ziatarov, A., and Kalcheva, D., *Biochem. Z.*, **284**, 12 (1936).

to the lactobacilli. Under our conditions *Escherichia coli* and hemolytic streptococci of Groups A and D were not affected by manganese.

The effect of manganese was only upon the rate of growth and not on the extent. With manganese, growth and acid production were complete in 12 to 16 hours; without it, approximately 40 hours incubation was required.

Effect of Mn⁺⁺ on Acid Production by Lactobacillus casei

| Mn ⁺⁺ | 0.1 N acid produced |
|------------------|---------------------|
| γ per cc. | cc. per 10 cc. |
| 0 | 4.8 |
| 0.26 | 7.8 |
| 1.30 | 9.2 |
| 2.6 | 9.4 |
| 13.0 | 9.0 |

An attempt was made to learn the mechanism of action of manganese by noting the effect on the rate of growth in substrates which are possible intermediates in the formation of lactic acid from glucose. Growth in hexose diphosphate was stimulated by manganese. Pyruvate or acetaldehyde and pyruvate supported only slight growth even in the presence of manganese and none in its absence. Other postulated intermediates were not available but it is believed that this approach to a study of the mechanism may prove useful.

EXCITATION OF INTRASPINAL MAMMALIAN AXONS BY NERVE IMPULSES IN ADJACENT AXONS

BY BIRDSEY RENSCHAW AND PER OLOF THERMAN

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(Received for publication, January 10, 1941)

Well-known experiments made with the rheoscopic nerve-muscle preparation reveal that the action currents of muscles can stimulate nerve fibers. It is less generally recognized that under certain conditions axons can be effectively stimulated by the activity of adjacent axons (Hering, 1882). Hering worked with the nerves of winter frogs. In one version of his experiment he prepared the peroneal and tibial nerves for stimulation, and observed the muscles innervated by other branches of the sciatic (fig. 1 a). For a few minutes after making a cut across the sciatic plexus, the delivery of a weak induction shock to the tibial and peroneal nerves was followed by a powerful contraction of the adductor muscles of the thigh. Hering made careful controls, which demonstrated that the motor axons of the adductor muscles were not excited by escape of the electrical stimulus applied to the primary (conditioning) axons, nor by spread of electrotonic changes. He was, therefore, led to believe that the impulses in the primary axons directly stimulated the secondary (tested) fibers.

Hering emphasized that the following conditions contributed to the success of his experiment: 1, the preparations were very excitable; 2, the two groups of fibers, conditioning and tested, converged and came to lie in intimate topographical association; 3, the common bundle containing the two groups of axons was freshly cut across. When these conditions are fulfilled, it is not difficult to perform a modification of Hering's experiment with frog nerves and to confirm his results (cf. also von Uexküll (1894) and Kwassow and Naumenko (1936)). Recently, several papers have reported the stimulation of hyperexcitable invertebrate axons by the activity of adjacent fibers (Jasper and Monnier, 1938; Katz and Schmitt, 1940; Arvanitaki, 1940a, b, c). In addition it has been shown that subthreshold alterations in the excitability of tested axons can be detected in experiments in which propagated secondary impulses are not initiated by impulses in neighboring conditioning axons (Otani, 1937; Katz and Schmitt; Blair and Erlanger, 1940).¹

¹Since the present paper was sent for publication, two additional papers on cross excitation between medullated axons have appeared (Feng and Li, Proc. Soc. Exper. Biol. and Med. 45: 870, 1940; Rosenblueth, This Journal 132: 119, 1941).

The intraspinal ascending branches of the afferent neurons of the mammalian spinal cord offer a preparation in which the conditions necessary for an experiment analogous to that of Hering are easily attained (fig. 1 b), since the ascending branches of adjacent dorsal root fibers lie in the same portion of the dorsal column. If one of two adjacent dorsal roots be stimulated and a fresh transection be made of the dorsal column cephalad to the root level, impulses passing up the column in the axons belonging to the stimulated root will excite in the column the axons belonging to the other root, and the impulses so set up may be recorded as a centrifugal volley in the latter. The central latency for the centrifugal impulses is so brief that the stimulation of the secondary axons can be effected only by processes contemporaneous with the spike potential in the primary axons.

Barron (1940) suggests that direct stimulation of the intraspinal branches of dorsal root fibers by impulses in adjacent axons may account for the centrifugal impulses of brief central latency which were observed in the dorsal roots by Matthews and himself (1935). In our experience, secondary impulses of very brief central latency have been observed only after section of the dorsal column.

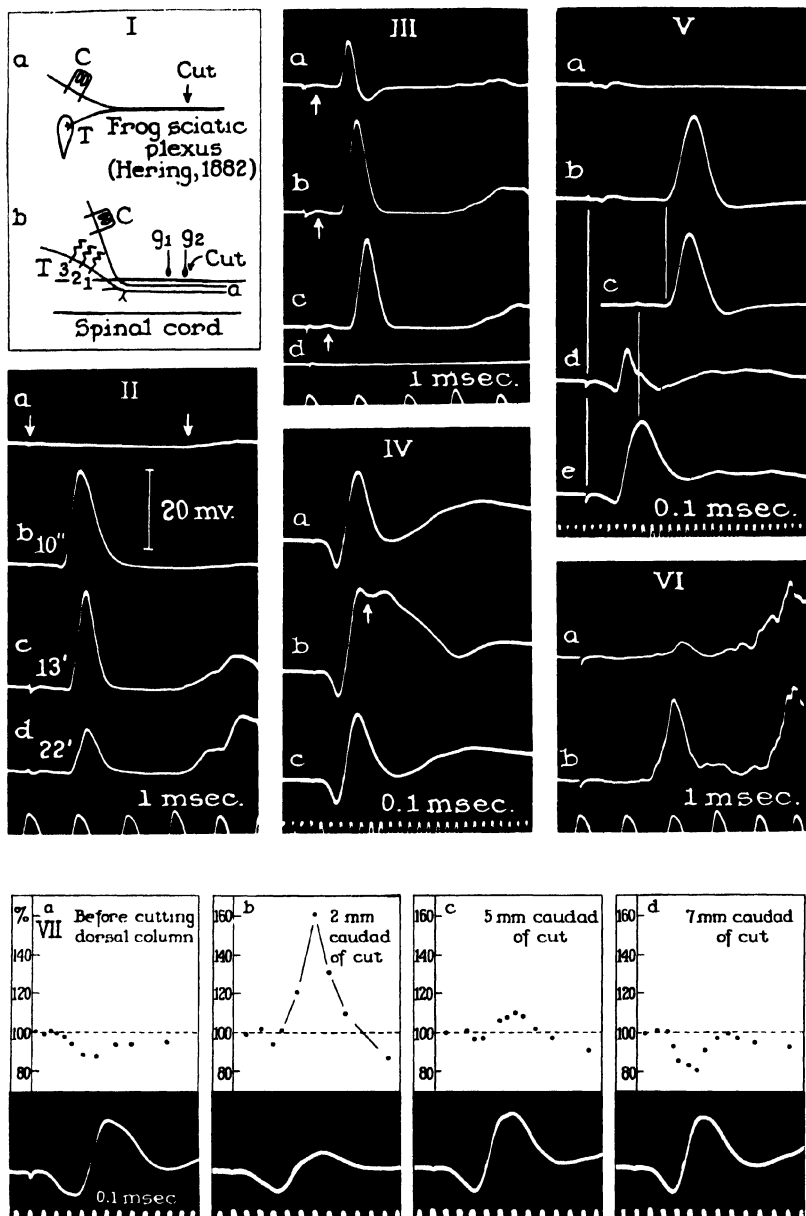
METHODS. The experiments were made on cats under Dial narcosis (Ciba, 0.6 cc./kgm.). In each experiment a laminectomy was performed and the dura opened. Usually two groups of lumbo-sacral dorsal rootlets were cut intradurally and prepared for stimulating (primary or conditioning group, C, fig. 1 b), and recording (secondary or tested group, T). In several experiments in which all dorsal roots were left intact, the sciatic nerve was stimulated just above the knee and records were taken from the sural nerve in the popliteal space. In some cases the reflex discharges evoked in the ventral roots by stimulation of the conditioning dorsal rootlets were examined. Action currents of the dorsal column axons were recorded, and these axons were also directly stimulated, through small Ag-AgCl electrodes placed upon the dorsum of the cord (g_1 and g_2 , fig. 1 b). The preparations were covered with paraffin oil to a depth of about one centimeter, in order to help maintain the cord and its roots in good condition for long periods of time. The customary differential amplifier and stimulating apparatus were used.

RESULTS. Stimulation of a group of dorsal rootlets (C, fig. 1 b) with a single shock maximal for alpha fibers produced in adjacent rootlets, T, the dorsal root reflex (fig. 2 a). As Toennies (1938) and Hursh (1940) have shown, the central latency for this discharge varies between 2.1 and 3.5 msec., depending upon the temperature of the preparation. There has been no indication that centrifugal impulses emerged from *uninjured* cords after shorter latencies. After section of the dorsal column cephalad to the point of entry of the conditioning and tested dorsal root fibers,

however, a striking change appeared in the oscillograms. A conspicuous deflection, considerably preceding that caused by the dorsal root reflex, occurred (fig. 2 *b*, *c* and *d*). Controls demonstrated that this deflection was due to impulses which were conducted centrifugally in the secondary fibers (fig. 3). The response was diphasic when led from two electrodes on the live tested fibers (fig. 3 *a*), and monophasic when the tested rootlets were crushed under the distal electrode (fig. 3 *b*). Further, the latency increased as the proximal lead was moved distally on the tested axons, and its size did not decrease rapidly, as would have occurred if the response were being led electrotonically from the cord. The possibility that the secondary fibers were stimulated intracentrally by spread, either of the stimulating current or of electrotonic changes in the conditioning axons, was tested and excluded by reversing the stimulating leads (fig. 3 *c*); the early centrifugal impulses were then still present and bore the same temporal relation to a small deflection (marked with arrow), which indicated the arrival of the conditioning volley at the cord. No significant current flowed from the stimulating transformer through the preparation to ground, for there was no response when one of the stimulating leads was disconnected from the preparation (fig. 3 *d*).

Additional experimental support exists for the contention that the early volley of centrifugal impulses is not dependent upon the proximity of the stimulating and recording electrodes to the cord or to the region of transection of the ascending axons. 1. The early centrifugal volley has appeared when the level of transection was as much as 40 mm. cephalad to the level of root entry. Its latency was then greater than when the cord was cut nearer the level of root entry. The increase in latency could be accounted for by the greater distances of conduction in the primary and secondary axons. 2. In four experiments the sciatic nerve was stimulated just above the knee and records were taken from the sural nerve, which contains only sensory fibers. When the spinal cord was intact, only the impulses of the dorsal root reflex appeared in the sural nerve. After transection of the dorsal column at the level of the 6th or 7th lumbar segment, the electrogram of the sural nerve showed, in addition to the dorsal root reflex, an earlier deflection comparable to that of figure 2 *c*. It must be concluded that the tested axons were excited intracentrally as a result of the activity of adjacent conditioning axons.

As is shown by the records of figure 2, the number of tested fibers excited by a given conditioning volley decreased progressively with the time that had elapsed after the transection of the dorsal column. With some favorable, cool preparations, such as that from which the records of figure 2 are taken, the volley in the secondary fibers initially amounted to as much as 25 millivolts and involved more than one-half of the alpha fibers of the tested rootlets; and centrifugal impulses could still be detected as much as



Figs. 1-7

Fig. 1 *a*. Diagram of Hering's experiment. The fibers of the peroneo-tibial nerve (*C*) intermingle in the sciatic plexus with the axons that supply the adductor muscles of the thigh (*T*). For a short time after cutting across the sciatic plexus, stimulation of the peroneo-tibial nerve at the knee initiated contractions of the adductor muscles. *b*. Diagram for the present experiments. The ascending branches (*a*) of adjacent dorsal rootlets (*C*, *T*) lie in close topographical association in the dorsal columns of the spinal cord. For a period of time after transection of the dorsal columns at g_2 , a centrifugal volley, which entered the cord over the fibers of one group of dorsal rootlets (*C*), served to initiate a volley of centrifugal impulses in the fibers of adjacent dorsal rootlets (*T*). Activity in the dorsal columns was recorded *via* electrodes (g_1 , g_2) placed on the dorsum of the cord. g_2 was located at the level of transection, g_1 about 2.5 mm. caudad of g_2 .

Fig. 2. The stimulation of dorsal column axons by impulses in adjacent axons. Conditioning dorsal rootlets: first sacral (S_1) and cephalic two-thirds of the 7th lumbar (L_7). Tested rootlets: caudal one-third L_7 . Record *a*, before transection of the ipsilateral dorsal column 5 mm. cephalad of L_7 . Record *b*, 10 seconds after transection; *c*, 13 minutes; *d*, 22 minutes. The amplification for records *a* and *b* is indicated by the voltage calibration on the figure; record *c*, 5 \times ; record *d*, 25 \times the amplification of *b*. The first arrow marks the escape of the conditioning shock; the second arrow indicates the onset of the dorsal root reflex discharge. Rectal temperature, 36.6°. Time as indicated.

Fig. 3. Same experiment as figure 2. The tested axons were alive under electrodes 1 and 2, killed under 3 (fig. 1 *b*). Record *a*, the diphasic response recorded from electrodes 1 and 2. Record *b*, monophasic response recorded from 1 and 3. Record *c*, as *b*, but stimulating leads reversed so that the cathode was in the distal rather than in the usual proximal position. The arrow marks a small deflection which is referable to the arrival of the conditioning volley at the cord. Record *d*, one stimulating lead disconnected from the preparation. Time as indicated.

Fig. 4. Impulses in the conditioning and tested fibers, as recorded from the dorsal column. The records are from electrode g_1 (fig. 1 *b*) placed about 2.5 mm. caudad of the level of transection and an indifferent electrode. Stimulated (*C*) dorsal rootlets: cephalic one-half L_7 . Record *a* was taken before the dorsal column was transected; record *b* immediately after transection; record *c*, about 20 minutes later. The deflection marked by the arrow in record *b* is due to impulses initiated in secondary axons by the impulses in the ascending branches of the conditioning L_7 dorsal root fibers. This deflection and the activity it represented had largely disappeared several minutes later (record *c*). Negativity at g_1 is recorded as an upward deflection. Rectal temperature, 36°. Time as indicated.

Fig. 5. Conditioning dorsal roots: S_1 and cephalic two-thirds L_7 . Tested dorsal rootlets: caudal one-third L_7 . Records *a*, *b*, and *c* are from the tested dorsal rootlets (*T*, fig. 1 *b*). Records *d* and *e* are from the dorsal column (bipolar leads from g_2 at the level of the section and g_1 2.5 mm. caudad to it). The stimulus for records *a*, *b*, *d* and *e* was a shock applied to the *C* rootlets; for record *c* a cathodal shock was delivered to the dorsal column at electrode g_1 . Records *a*, *c* and *d* were obtained before transection of the dorsal column 15 mm. cephalad of L_7 ; records *b* and *e* after the section had been made. Rectal temperature, 36.9°. Time as indicated.

Fig. 6. Effect of dorsal column section on motor discharge. The 7th lumbar dorsal root was stimulated and records were taken from the corresponding ventral root axons. Record *a* before, and record *b* 30 seconds after, a transection 15 mm. above L_7 . The transection involved little, if any, of the cord other than the dorsal column. Cool preparation. Time as indicated.

Fig. 7. Subthreshold excitability changes induced in tested axons by a condition-

half an hour later. In warm animals, as would be expected, the restitution of the cut axons proceeded more rapidly and the response usually disappeared within a few minutes or even seconds after the transection. Thus, although the response did occur when the dorsal columns were at temperatures normal for cats, it was more favorably examined in cooler preparations.

The temporal relation between the arrival of the conditioning impulses at the region near the cut and the initiation of impulses in the adjacent tested axons is of interest. Figures 5 *a* and *b* reveal that a conditioning centripetal volley which entered the cord in the fibers of a group of dorsal rootlets produced a centrifugal volley in adjacent rootlets after, but not before, transection of the dorsal column about 15 mm. cephalad to the level of root entry. Records *d* and *e* show the potential changes recorded between an electrode (g_2 , fig. 1 *b*) on the dorsal column at the level of transection and a second electrode (g_1) placed 2.5 mm. caudad to g_2 . Record *d* was made just before transection of the dorsal column and record *e* about 20 minutes later. Record *c* shows a volley initiated in the tested dorsal root fibers by a cathodal shock applied through electrode g_1 . The shock-response interval included a short utilization time, estimated to have been about 0.1 msec., and the conduction time from the region caudad of the cut to the recording electrodes. Examination of the oscillograms with reference to the simultaneous ordinates which have been drawn makes it clear that the tested fibers were excited at the time when the spike negativity of the conditioning axons existed at the region caudad to the cut. The results of a number of experiments have established that the centrifugal discharge arrived at the recording electrodes only 0.1 to 0.3 msec. later than it would have if the conditioning volley had travelled in an uninterrupted fiber path from the stimulating cathode cephalad to the region of the cut and back to the recording electrodes.

The fact that secondary axons were excited during the period of negativity due to the spike potential of the conditioning impulses is likewise

ing volley in adjacent axons. The testing stimulus was a submaximal cathodal shock applied at g_1 (fig. 1 *b*). The tested response was recorded through the electrodes on one-half of the L_7 dorsal rootlets (*T*). The conditioning activity was a volley in the other L_7 dorsal root fibers (*C*). The oscillograms are records of the potential changes which were set up at g_1 by the conditioning volley in isolation. Ordinates of graphs: $\frac{\text{height of conditioned tested response}}{\text{height of unconditioned tested response}} \times 100$. Abscissae: interval at which the testing shock followed the conditioning stimulus. *a*, before transection of the dorsal column; g_1 was about 15 mm. cephalad of the 7th lumbar segment. Similar curves were obtained for other axial positions of g_1 . *b*, *c* and *d*, after transection of the dorsal column about 16 mm. cephalad of the 7th lumbar segment. The distance of g_1 caudad of the cut is indicated on the figure for each curve.

demonstrated by an examination of the records of figure 4. The potential changes attributable to a conditioning volley were recorded from the electrode g_1 on the dorsal column and an indifferently placed electrode. Record *a* was taken before section of the dorsal column. Record *b* was obtained immediately after transection of the column about 2.5 mm. cephalad to g_1 , and record *c* about 20 minutes later. Records *a* and *c* show an initial deflection which is referable to the afferent volley; this is followed by the first part of the negative cord potential (Gasser and Graham, 1933). In record *b* an arrow marks an additional deflection which must be interpreted as caused by impulses that were set up in secondary fibers by impulses of the conditioning volley. In confirmation of the conclusion derived from the records of figure 5, the tested fibers were stimulated at approximately the time that the negativity due to the primary impulses was maximal a few millimeters caudad to the cut.

The negative cord potential is a sign of the activity of the spinal interneurons which are stimulated by a primary afferent volley (Gasser and Graham, 1933). Therefore, the activity of postsynaptic elements in the cord cannot be responsible for the excitation of the tested axons, because the secondary impulses are set up before the beginning of the negative cord potential (figs. 4 and 5).

Subthreshold changes in the excitability of the tested axons could be detected even after the conditioning volley had ceased to initiate secondary impulses. These subliminal excitability changes were measured as follows: A cathodal shock applied through electrode g_1 (fig. 1 *b*) stimulated the ascending branches of some of the dorsal root fibers labelled *T* in figure 1 *b*. The impulses travelled caudally and emerged as a submaximal centrifugal volley in the fibers *T*. The changes in the size of this volley, which were induced by a preceding conditioning volley ascending from rootlets *C*, served as measures of the excitability changes of the tested axons.

The observed excitability changes are in complete accord with the changes observed in frog nerves by Blair and Erlanger (1940). So long as the dorsal column remained intact and uninjured, the excitability of the tested axons was decreased during the period of spike negativity in the adjacent conditioning axons (fig. 7 *a*). Transection of the dorsal column produced a complete change in the excitability curves determined at regions close to the cut. The data of figures 7 *b*, *c* and *d* were obtained several minutes after the cut had been made—after the conditioning volley had ceased to initiate secondary impulses. The oscillogram and curve of figure 7 *b* were obtained with g_1 at a point 2 mm. caudad to the cut. A large increase of the excitability of the tested axons occurred during the period of relative negativity which was produced by the conditioning impulses. Five millimeters caudad to the cut the increase of excitability

was much less (fig. 7 c); and at 7 mm. (fig. 7 d) the excitability curve approached that which obtained before injury.

Thus the greatest increase in the excitability of the tested axons occurred 1, at regions a short distance ($2 \pm$ mm.) caudad to the cut; and 2, at the time when the negativity due to the conditioning impulses was greatest at this locus. These findings are in accord with the fact that, when the tested axons were effectively stimulated by the conditioning volley, the secondary impulses arose at approximately the time the conditioning volley produced the greatest relative negativity a short distance caudad to the transection (figs. 4 and 5).

Figure 6 shows that reflex discharges, evoked in a lumbar ventral root by stimulation of a group of dorsal rootlets, were greatly augmented immediately after section of the ipsilateral dorsal column. A few minutes after the transection had been made, the reflex had reverted to approximately its original size. These findings were not unexpected, because for a short while after transection of the dorsal column the reflexogenic action of an afferent volley must be supplemented by the effects of impulses in secondarily excited afferent neurons. Thus the direct excitation of dorsal column axons by impulses in adjacent axons is one of the factors responsible for the immediate increase in spinal reflexes which is induced by cord section (Sherrington and Sowton, 1915; Forbes, Cobb and Cattell, 1923). It is clear that additional factors must be involved when complete transection of the cord induces an increase that persists for prolonged periods of time.

DISCUSSION. The present experiments with intraspinal mammalian axons confirm Hering's (1882) original findings for frog nerves. The controls which have been made in both instances demonstrate that the excitation of the tested axons is not to be explained as an artefact caused by stimulus escape or by the spread of electrotonic changes from the stimulating electrodes to the tested axons (cf. the "paradoxical contraction" of duBois-Reymond, 1849). Hering presumed that the tested axons were stimulated by the "negative variation" of the primary axons. The present experiments prove the likelihood of this supposition, because they show that secondary impulses arise approximately at the time the relative negativity due to the conditioning impulses attains its maximal value near the transection (figs. 4 and 5). This point deserves emphasis because a different result has been obtained in experiments with unmyelinated invertebrate axons (Jasper and Monnier, 1938; Arvanitaki, 1940a, b, c).

Subthreshold changes in the excitability of medullated frog axons are induced by impulses in adjacent fibers (Blair and Erlanger, 1940). Blair and Erlanger find that, near the region of a cut or an injury, the excitability of tested fibers is increased by the arrival of conditioning impulses in adjacent axons. Figure 7 reveals that comparable changes occur in the

tested intraspinal axons. Hering's experiments and the present results show that this increased excitability sometimes attains threshold, and impulses are initiated.

The direct excitation of tested axons by the action currents of adjacent axons has been observed only after section of or injury to the common bundle of conditioning and tested axons. There are two apparent reasons why the proximity of a region of fresh injury might facilitate the stimulation of the tested axons. First, the external electric field which the conditioning impulses produce as they approach the region of the cut is altered, so that it may be a more effective stimulus (fig. 5 *d* and *e*). Second, the excitability of regions of the tested axons adjacent to the cut is temporarily increased after the production of the injury (Hering).

Since under certain circumstances the action current of axons can effectively stimulate other, anatomically independent axons, it is obvious that a possible anatomical discontinuity at synapses offers no *a priori* reason for assuming that the action currents of pre-synaptic fibers and endings could not excite post-synaptic neurons.

It seems likely that the excitability of neurons in the central nervous system may depend not only upon the effects produced by the arrival of impulses at synapses, but also by the environmental changes produced by the activity of neighboring neurons. As Grundfest (1940) has suggested, the excitability changes produced in axons by the activity of adjacent axons are, therefore, of interest as examples of the effects that may occur in the more complex systems.

SUMMARY

The dorsal column of the spinal cord contains the ascending branches of sensory fibers which enter the cord over the ipsilateral dorsal roots. For a period of time after transection of the dorsal column, at a level cephalad to the entry of a stimulated dorsal root, impulses in the ascending branches of the active fibers directly excite adjacent axons. The impulses in the secondary axons then travel antidromically (caudally) and emerge as a centrifugal discharge in dorsal root fibers adjacent to those which carried the centripetal volley. The secondary impulses are initiated by processes contemporaneous with the arrival of primary impulses at the region caudad to the cut, and before post-synaptic spinal neurons become active.

Subthreshold increases in the excitability of tested dorsal column axons are produced by a primary volley which does not actually initiate secondary impulses. The increase in excitability is greatest a few millimeters caudad to a cut. At this locus the maximal excitability coincides with the time at which the conditioning impulses produce the greatest relative negativity. Before section of the dorsal column, the excitability of tested axons is decreased by impulses conducted in adjacent axons.

Transection of the dorsal column produces an immediate increase in the size of the motor discharges that are evoked by dorsal root volleys.

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THE SULFHYDRYL GROUPS OF EGG ALBUMIN*

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INTRODUCTION

The first part of this paper describes a number of ways of estimating the SH groups of denatured egg albumin by measuring how much of a particular SH reagent is needed to abolish the SH groups and by measuring how much ferricyanide is reduced by the SH groups. The same titration value is obtained whether the SH groups are abolished by the oxidizing agents, ferricyanide and tetrathionate, or the heavy metal compound, *p*-chloro-mercuribenzoate; whether the titration is carried out in a guanidine hydrochloride solution or in a solution of Duponol PC, a detergent consisting of long chain alkyl sulfates; whether the abolition of the nitroprusside test or the reduction of ferricyanide is used as proof of the abolition of the SH groups. The same amount of ferricyanide is reduced by denatured egg albumin whether the reduction is carried out in a solution of guanidine hydrochloride, urea, or of Duponol PC. This agreement between the SH values obtained by very different procedures is strong evidence of the validity of the results.

Ferricyanide is a particularly convenient titrating agent. It is readily available and stable. Under the conditions used ferricyanide reacts almost immediately with the SH groups of denatured egg albumin and yet does not react with other protein groups. Altogether, denaturation by guanidine hydrochloride or Duponol PC and oxidation of the SH groups by ferricyanide can be carried out in a few minutes.

It is important to use reagents of suitable purity for the titrations in guanidine hydrochloride solution. I have found that some samples of guanidine hydrochloride and of protein contain impurities which bring about the abolition of SH groups and thus interfere with the nitroprusside test and the SH titrations in guanidine hydrochloride solution. A method has accordingly been worked out for obtaining pure guanidine hydro-

* A brief account of the SH titration methods has already been published (Anson, 1940 b).

chloride, and the techniques of the nitroprusside test and the SH titrations in guanidine hydrochloride solution have been so modified as to minimize interference by impurities.

Whether or not the SH groups of native egg albumin react with a particular SH reagent depends on which SH reagent is used. All the SH groups of egg albumin can be abolished by reaction of the native form of the protein with iodine and some can be abolished by reaction of the native form of the protein with iodoacetamide (Anson, 1940 *a*) despite the fact that the SH groups of native egg albumin do not give a pink color with nitroprusside (Heffter, 1907; Arnold, 1911), and are not oxidized by cystine (Mirsky and Anson, 1935), ferricyanide (Mirsky and Anson, 1936), or porphyrindin (Kuhn and Desnuelle, 1938). The present experiments show further that *p*-chloromercuribenzoate, which combines firmly with the SH groups of denatured egg albumin, combines with native egg albumin either not at all or at least much more loosely than it combines with denatured egg albumin.

The reactions of iodine and native egg albumin (Anson, 1940 *a*) have now been studied in more detail. It has been found that if a small amount of iodine is added in the cold, the SH groups of neutral native egg albumin are all abolished without oxidation of many of the SH groups beyond the S-S stage and without conversion of many tyrosine groups into di-iodotyrosine groups. If enough iodine is added, the SH groups are oxidized beyond the S-S stage, the S-S groups originally present are oxidized, and the tyrosine groups are converted into di-iodotyrosine groups.

The present pictures of protein structure are not complete enough to provide detailed explanations of the various reactions of the SH groups of different native and denatured proteins with different SH reagents. The facts about the properties of protein groups such as SH groups, however, are important for the development of an adequate theory of protein structure.

The SH titrations in guanidine hydrochloride solution which were worked out with egg albumin can be applied to tobacco mosaic virus. Furthermore, the SH groups of the virus, like the SH groups of egg albumin, can be abolished by reaction of the native form of the protein with iodine. No reaction other than the iodine reaction is known by which the SH groups of native egg albumin and tobacco mosaic virus can be abolished. The discovery of the iodine reaction has thus made possible the study of the biological properties of tobacco mosaic virus which has been modified by oxidation of its SH groups by iodine. The chemical and biological experiments with tobacco mosaic virus, which were suggested by the experiments with egg albumin, will be described in other papers.

Previous Estimations of the SH Groups of Denatured Egg Albumin.—The various procedures which have been used to estimate protein SH groups (Mirsky and Anson, 1935; Kuhn and Desnuelle, 1938; Greenstein, 1938; Anson, 1939) are all similar in principle to the methods used to estimate the SH groups of simple SH compounds such as cysteine and glutathione. SH reagents in general react less readily with protein SH groups than with the SH groups of cysteine. Some SH reagents, furthermore, can, under suitable conditions, react with protein groups other than SH groups. The problem, therefore, in the estimation of the SH groups of unhydrolyzed protein is to find conditions under which the SH reagent reacts with all the protein SH groups and no other groups. These conditions have apparently been fulfilled in the estimation of the SH groups of egg albumin by two methods which were developed from the earlier work. In the first method, one measures how much porphyrindin has to be added to denatured egg albumin in guanidine hydrochloride solution so that all the protein SH groups are oxidized and the protein no longer gives a pink color with nitroprusside (Greenstein, 1938). In the second method, one measures how much ferricyanide is reduced by denatured egg albumin in a solution of the detergent, Duponol PC (Anson, 1939).

Ferricyanide and porphyrindin are added to denatured rather than to the native egg albumin because native egg albumin does not reduce ferricyanide and porphyrindin at all. Guanidine hydrochloride or Duponol PC are added because in the absence of such substances not all the SH groups even of denatured egg albumin are rapidly oxidized by dilute ferricyanide and porphyrindin. The nitroprusside test is carried out in guanidine hydrochloride solution but not in Duponol PC solution because the SH groups of denatured egg albumin give a strong pink color with nitroprusside in guanidine hydrochloride solution but only a negligible pink color in Duponol PC solution. The ferricyanide reduction can be carried out in guanidine hydrochloride and urea solution as well as in Duponol PC solution but the estimation of the ferrocyanide formed as Prussian blue is less convenient than when the reaction is carried out in Duponol PC solution.

Various tests were carried out by Greenstein and myself to show that ferricyanide and porphyrindin under the conditions used react specifically and completely with the SH groups of denatured egg albumin. Since no one of these tests is conclusive, the validity of the SH estimations has been tested by comparing the results obtained by different procedures.

SH Titrations in Guanidine Hydrochloride Solutions.—In the porphyrindin titration as originally carried out (Greenstein, 1938) guanidine hydrochloride is added to a neutral protein solution, the solution is allowed to stand

45 minutes, porphyrindin is added to the protein denatured by the guanidine hydrochloride, and finally nitroprusside and ammonia are added to see whether enough porphyrindin has been added to oxidize all the SH groups.

The new titrations in guanidine hydrochloride solution are carried out as follows. To 0.5 cc. of 2 per cent native egg albumin or tobacco mosaic virus there are added 0.1 cc. of neutral phosphate buffer, 0.5 cc. of ferricyanide, tetrathionate or *p*-chloromercuribenzoate¹ solution, and 1.2 gm. of guanidine hydrochloride of tested purity. 3 minutes later test is made for the abolition of the SH groups either by seeing whether the protein gives a nitroprusside test in the presence of dilute cyanide or by seeing whether the protein can still reduce ferricyanide in Duponol PC solution. The concentration of titrating agent is found which just suffices to abolish the SH groups.

The new titrations differ from the porphyrindin titration in that different titrating agents are used; interference by impurities is minimized by using especially purified guanidine hydrochloride, adding the titrating agent before the guanidine hydrochloride, and carrying out the nitroprusside test in the presence of cyanide; the ferricyanide reduction test as well as the nitroprusside test is used to prove the abolition of SH groups; and the whole titration is carried out in the presence of phosphate buffer. I shall now discuss the reasons for the changes which have been made.

In the new titrations ferricyanide, tetrathionate, and *p*-chloromercuribenzoate are used as titrating agents instead of porphyrindin. The substitution of the ferricyanide and tetrathionate for porphyrindin makes the titration in guanidine hydrochloride solution safer and much more convenient. Porphyrindin is hard to prepare, unstable, and a dangerously strong oxidizing agent. Although porphyrindin reacts first with the SH groups of denatured egg albumin in guanidine hydrochloride solution it also in time reacts with other groups. Ferricyanide and tetrathionate are readily available and are weaker oxidizing agents than porphyrindin.

The inclusion of mercuribenzoate as a titrating agent provides a good test for the SH specificity of the titration. Whereas porphyrindin, ferricyanide, and tetrathionate oxidize SH to S-S, mercuribenzoate combines with but does not oxidize SH groups. It is conceivable that the oxidizing agents might oxidize protein groups other than SH groups or that mercuribenzoate might combine with groups other than the SH groups. The SH group, however, is the only protein group known to react with both oxidizing agents and heavy metal compounds.

Formaldehyde abolishes the SH groups of denatured egg albumin in guanidine hydrochloride solution only if the formaldehyde is added in great excess. Formaldehyde cannot, therefore, be used as a titrating agent.

The following observations show that some samples of guanidine hydrochloride contain impurities which bring about the abolition of SH groups and so interfere with the

¹ *p*-chloromercuribenzoate has been used as an SH reagent by Hellerman (1937; 1939).

nitroprusside test and that this interference can be diminished by cyanide. 5 mg. of denatured egg albumin gives a strong pink color with nitroprusside in guanidine hydrochloride solution. I have found, however, that the pink color obtained is much stronger with some samples of commercial guanidine hydrochloride (Eastman or Hoffman-La Roche) than with others. When a guanidine hydrochloride is used which gives a weak color, then the color is weaker the more guanidine hydrochloride is used and the longer the denatured protein is allowed to stand in guanidine hydrochloride solution before the addition of nitroprusside. A sample of guanidine hydrochloride which gives a weak color gives a strong color if it is first recrystallized. If 1 drop of 0.1 N cyanide is added to the protein solution before the addition of guanidine hydrochloride, then a strong nitroprusside test is obtained with all samples of guanidine hydrochloride. Even when a strong nitroprusside test is obtained without cyanide, cyanide slows up the rate of fading of the pink color. On the other hand, the rate of fading can be enormously increased by adding an amount of copper sulfate equivalent to only 10 per cent of the SH groups present. The results which have been summarized do not definitely prove how the impurities in guanidine hydrochloride bring about the abolition of SH groups. They suggest, however, that the impurities are in part, at least, heavy metal compounds which catalyze the oxidation of the SH groups of denatured egg albumin by oxygen and that cyanide inhibits this oxidation of protein SH groups by oxygen by combining with the heavy metal impurities. It is known that heavy metal compounds can catalyze the oxidation of the SH of cysteine (references in Bernheim and Bernheim, 1939) and of denatured egg albumin (Rosenthal and Voegtlin, 1933) by oxygen.

The cyanide added to diminish the effects of impurities in the nitroprusside test is too small in amount to cause any appreciable reduction of S-S to SH. Cystine and denatured egg albumin whose SH groups have been oxidized to S-S groups do not give any color with nitroprusside in guanidine hydrochloride solution even when 1 drop of 0.1 N cyanide is added. The ease with which the S-S groups of a denatured protein are reduced by cyanide varies from protein to protein. 1 drop of 0.1 N cyanide is safe for those proteins which I have tried, but it may not be safe for all proteins.

The following experiments show that impurities in guanidine hydrochloride reduce the amount of the ferricyanide needed to abolish the SH groups of denatured egg albumin, and that the effect of impurities is much less in the new titration procedure in which the titrating agent is added before the protein is denatured by guanidine hydrochloride than in the old titration procedure in which the protein is allowed to stand in guanidine hydrochloride solution before the addition of the titrating agent. When satisfactory guanidine hydrochloride is used the amount of ferricyanide needed to abolish the nitroprusside test is the same whether the ferricyanide is added before the addition of guanidine hydrochloride or 30 minutes after the addition of guanidine hydrochloride. With one poor sample of Eastman guanidine hydrochloride (not the worst), nine-tenths the normal amount of ferricyanide was required to abolish the nitroprusside test if the ferricyanide was added before the addition of guanidine hydrochloride, but only five-tenths the normal amount if the ferricyanide was added 30 minutes later.

The discrepancy between the amount of ferricyanide needed to abolish the SH groups of egg albumin when the ferricyanide is added before the guanidine hydrochloride and when it is added 30 minutes after the guanidine hydrochloride can be used as a test for the purity of the guanidine hydrochloride. When such a test is applied to commercial guanidine hydrochloride, usually, in my experience, the product is found to be unsatis-

factory. Guanidine hydrochloride cannot be purified by recrystallization without great loss. I have therefore found conditions for the effective and economical purification of guanidine carbonate. Guanidine hydrochloride prepared from purified guanidine carbonate is satisfactory for SH titrations.

Greenstein (1938) found that the SH groups of his egg albumin were stable in neutral guanidine hydrochloride solution. His sample of guanidine hydrochloride was therefore satisfactory.

Although one can always make sure that one has pure guanidine hydrochloride and pure egg albumin, not all proteins can readily be obtained in as pure a state as egg albumin. With some proteins it is particularly important to have a titration procedure like the present one which minimizes the effects of impurities and to carry out tests for the presence of impurities. The origin of the present detailed experiments with guanidine hydrochloride, in fact, was my inability to obtain a constant value for the SH content of tobacco mosaic virus when different samples of guanidine hydrochloride and virus were used.

The same titration value is obtained if the nitroprusside test on the albumin treated with the titrating agent is carried out almost immediately after the addition of the titrating agent and the guanidine hydrochloride or 30 minutes after the addition of the titrating agent and guanidine hydrochloride. The waiting has therefore been eliminated and the time needed for the titration very much shortened.

Instead of using the disappearance of the nitroprusside test as an indication that all the SH groups have been abolished one can use the failure to reduce ferricyanide. After the titrating agent and guanidine hydrochloride have been added to the protein, the protein is precipitated and washed with trichloroacetic acid, the precipitate is dissolved in neutral Duponol PC solution, ferricyanide is added, and a test is made for ferrocyanide. If either the nitroprusside test or the ferricyanide test for SH were insensitive or not specific for SH then different titration values would be obtained by using these two very different SH tests for the end point. Since the nitroprusside test is more convenient than the ferricyanide reduction test if the titration is carried out in guanidine hydrochloride solution, the ferricyanide reduction test is used not as a routine procedure but only as a check on the validity of the titrations.

The whole SH titration is in all cases carried out in a neutral solution buffered with phosphate. Phosphate establishes a reproducible pH. In the absence of phosphate, furthermore, a pink color is formed immediately on the addition of nitroprusside to a neutral or even slightly acid guanidine hydrochloride solution of denatured protein and the color becomes stronger on the subsequent addition of ammonia. Phosphate conveniently prevents the formation and fading of the pink color before the addition of ammonia.

Cyanide must not be present during the titrations with ferricyanide or mercuribenzoate despite the fact that it is desirable to have cyanide present during the nitroprusside test. For cyanide combines with mercuribenzoate and inhibits the oxidation of protein SH groups by ferricyanide. The mechanism of this inhibition has not been studied. It is possible that the oxidation of protein SH groups by ferricyanide is catalyzed by heavy metal impurities which combine with cyanide. Dilute cyanide does not prevent

the oxidation of the SH groups of denatured egg albumin in neutral guanidine hydrochloride solution by tetrathionate. (Since submitting this paper I have found that cyanide inhibits almost completely the oxidation of SH groups by ferricyanide, tetrathionate, and the uric acid reagent provided the concentration of cyanide is high enough and that copper and zinc ions promote these oxidations.)

In agreement with the results previously obtained by the porphyrindin titration method the equivalent of 1 cc. of 0.001 M ferricyanide, tetrathionate, or mercuribenzoate is needed to abolish the SH groups of 10 mg. denatured egg albumin in guanidine hydrochloride solution whether the abolition of the nitroprusside test or the failure to reduce ferricyanide is used as an end point. It should be emphasized that the agreement between the new titrations and the porphyrindin titration in its original form exists only when the samples of protein and guanidine hydrochloride used happen to be free of impurities which interfere with the original titration method much more than they do with the new methods.

Rate and Completeness of SH Reactions in Neutral Guanidine Hydrochloride Solution.—As we have seen, if 1 cc. of 0.001 M ferricyanide, tetrathionate, or mercuribenzoate is added to 10 mg. of denatured egg albumin in neutral solution and ammonia and nitroprusside are added 3 minutes later, no pink color is obtained. The question arises, does the SH reagent react with the protein SH groups in the neutral solution, or after the addition of ammonia, or is the colored compound of the nitroprusside test formed but destroyed by the SH reagent before it can be observed? Ferricyanide and mercuribenzoate can rapidly destroy the color formed in the nitroprusside reaction. The experiments designed to answer this question show that the abolition of the SH groups in neutral solution by ferricyanide and mercuribenzoate is completed in 3 minutes but that part of the tetrathionate reaction takes place after the addition of ammonia.

The following experiments show that the oxidation of the SH groups by ferricyanide takes place in the neutral solution. After the addition of the ferricyanide to the neutral protein solution the solution is colorless, indication of reduction of the brown ferricyanide to the colorless ferrocyanide. If the protein which has been treated with ferricyanide is precipitated and washed with trichloroacetic acid, a protein precipitate is obtained which is free of ferricyanide and has been exposed to ferricyanide in neutral but not in alkaline solution. The SH groups of this protein have been abolished. The protein gives no nitroprusside test when dissolved in guanidine hydrochloride solution and does not reduce ferricyanide in neutral Duponol PC solution.

Further experiments show that tetrathionate, like ferricyanide, can oxidize all the SH groups of denatured egg albumin in neutral solution but that the oxidation by tetrathionate is slower than the oxidation by ferricyanide and so is not complete in 3 minutes.

The trichloroacetic acid precipitate of the albumin treated for 3 minutes with tetrathionate in neutral guanidine hydrochloride solution still gives a moderately strong nitroprusside test in guanidine hydrochloride solution and reduces about half as much ferricyanide in Duponol solution as untreated albumin. Since no nitroprusside test is obtained if ammonia and nitroprusside are added directly after 3 minutes, part of the tetrathionate reaction responsible for the abolition of the nitroprusside test must take place after the addition of ammonia. If the tetrathionate is allowed to stand 30 minutes in the neutral guanidine hydrochloride solution before the addition of trichloroacetic acid, then it is found that the SH groups have all been abolished by the reaction in neutral solution.

Finally, the evidence that mercuribenzoate combines with the SH groups of denatured egg albumin in neutral guanidine hydrochloride solution. If first 1 cc. of 0.001 M mercuribenzoate and then 1 cc. of 0.001 M ferricyanide are added to 10 mg. of denatured egg albumin in neutral guanidine solution, the brown color of the ferricyanide persists. If the mercuribenzoate had not combined with and protected the SH groups, the ferricyanide would have been reduced to colorless ferrocyanide. When mercuribenzoate combines with SH groups of urease it similarly protects them from oxidation by porphyrindin (Hellerman, 1939).

The compound between mercuribenzoate and the SH groups of denatured egg albumin in neutral guanidine hydrochloride solution is dissociated by trichloroacetic acid. If denatured egg albumin which has combined with mercuribenzoate is precipitated with trichloroacetic acid and dissolved again with guanidine hydrochloride it gives about as strong a nitroprusside test as egg albumin which has never been exposed to mercuribenzoate.

Measurement of Ferricyanide Reduction in Guanidine Hydrochloride and Urea Solution.—1 cc. of 0.001 M ferrocyanide is formed when ferricyanide is reduced by 10 mg. of denatured egg albumin in Duponol PC solution (Anson, 1940 a) or, as in the present experiments, in guanidine hydrochloride or urea solution. The amount of ferrocyanide formed is within wide limits independent of the ferricyanide concentration.

The estimation of SH groups by ferricyanide reduction is more convenient in Duponol PC than in guanidine hydrochloride or urea solution. Duponol PC, unlike guanidine hydrochloride and urea, prevents the precipitation of denatured egg albumin by the acid ferric sulfate added for the estimation of ferrocyanide as Prussian blue. Duponol PC interferes with the development of Prussian blue less than guanidine hydrochloride and in neutral solution denatures egg albumin more rapidly than urea.

Denaturation in neutral urea solution is slow and egg albumin loses some of its SH groups on standing in neutral urea solution if ordinary commercial urea is used and the urea solution does not contain cyanide. In the present experiments, therefore, denaturation by urea is brought about in acid solution in which denaturation is rapid and SH groups are more stable. When the acid urea solution containing 10 mg. of denatured egg albumin is neutralized, 1 cc. of 0.001 M ferricyanide is added. 1 cc. of 0.001 M ferrocyanide is formed and the protein when precipitated with trichloroacetic acid and redissolved with guanidine hydrochloride gives no nitroprusside test. The nitroprusside test of untreated egg albumin in guanidine hydrochloride solution is much more intense than the test in urea solution.

I have not been able to confirm the conclusion of Greenstein (1938) that urea "liberates" fewer SH groups from egg albumin than guanidine hydrochloride. Even if 1 cc. of 0.001 M ferricyanide and urea are added to a neutral solution of 10 mg. of egg albumin which has not been treated with acid, the protein after being precipitated by trichloroacetic acid no longer gives a pink color with nitroprusside in guanidine hydrochloride solution.

As will be described elsewhere, the SH groups of egg albumin can be estimated by the blue color given with the uric acid reagent, the SH value being the same as that obtained by the present methods. When unhydrolyzed albumin is used the reaction is carried out in urea solution. When albumin partially hydrolyzed by pepsin or acid is used, the presence of urea is not necessary.

Urea promotes the oxidation not only of the SH groups of denatured egg albumin but also the oxidation of free cysteine, tyrosine, and tryptophane. Partial hydrolysis "activates" not only SH groups but the few other protein groups I have tried.

SH Titrations in Duponol PC Solution.—In neutral Duponol PC solution as in neutral guanidine hydrochloride solution 1 cc. of 0.001 M ferricyanide, tetrathionate, or mercuribenzoate is required to abolish the SH groups of 10 mg. of denatured egg albumin.

After the SH groups of denatured egg albumin have been abolished by ferricyanide or tetrathionate, the protein when precipitated by trichloroacetic acid and redissolved in guanidine hydrochloride solution no longer gives a nitroprusside test. As in guanidine hydrochloride solution, the ferricyanide reaction is more rapid than the tetrathionate reaction and cyanide interferes with the ferricyanide reaction but not with the tetrathionate reaction.

After mercuribenzoate has combined with all the SH groups of denatured egg albumin in neutral Duponol PC solution the protein no longer reduces dilute ferricyanide. Thus when the SH estimation is carried out in Duponol PC solution as when it is carried out in guanidine hydrochloride solution, it is possible to titrate the SH groups with both an oxidizing agent and a heavy metal compound and to use both the nitroprusside reaction and the ferricyanide reduction as tests for the abolition of the SH groups.

The ferricyanide reduction test should not be used after the tetrathionate reaction because the decomposition products formed from tetrathionate in acid solution reduce ferricyanide. The nitroprusside test should not be used after the mercuribenzoate reaction because trichloroacetic acid dissociates the compound between mercuribenzoate and SH.

The Reactions of Iodine and Native Egg Albumin.—Despite the fact that native egg albumin does not react with nitroprusside, ferricyanide, or porphyrindin, all the SH groups of egg albumin can be abolished by reaction of the native form of the protein with iodine. Native egg albumin which has reacted with iodine no longer gives a nitroprusside test when denatured

nor does it reduce ferricyanide in Duponol PC solution (Anson, 1940 *a*). I have now studied the reactions between iodine and neutral native egg albumin in somewhat more detail, mainly in order to compare the egg albumin reactions with the reactions between neutral native tobacco mosaic virus and iodine, which will be described elsewhere.

By adding iodine in acid solution it is possible to oxidize the SH groups of native egg albumin without converting the tyrosine groups into di-iodotyrosine groups. Iodine abolishes the SH groups of native egg albumin even at pH 3.2 (Anson, 1940 *a*). At pH 3.2 iodine does not react with free tyrosine, or with the proteins pepsin (Herriott, 1937), and chymotrypsinogen (Anson, 1940) which contain tyrosine but not cysteine. The present experiments show that it is also possible in neutral solution to oxidize the SH groups of native egg albumin without converting many tyrosine groups to di-iodotyrosine groups or oxidizing many of the SH groups beyond the S-S stage.

If 1.3 cc. of 0.001 *N* iodine is added to 10 mg. of native egg albumin at 0°C., all the iodine is absorbed as shown by a negative starch test. All the SH groups are abolished as shown by a negative nitroprusside test in guanidine hydrochloride solution. Theoretically it takes 1 cc. of 0.001 *N* iodine to oxidize the SH of 10 mg. of egg albumin to S-S. The excess 0.3 cc. of 0.001 *N* iodine actually added is not sufficient to cause much further oxidation of the sulfur groups to RSOH, RSO₂H, or RSO₃H, or to convert many tyrosine groups into di-iodotyrosine groups. The 10 mg. of egg albumin treated with 1.3 cc. of 0.001 *N* iodine still gives a strong nitroprusside test in guanidine hydrochloride solution, if the protein is exposed to strong cyanide alkaline guanidine hydrochloride solution before the addition of nitroprusside, indicating S-S groups which are reduced to SH by alkaline cyanide. The 10 mg. of egg albumin which has absorbed 1.3 cc. of 0.001 *N* iodine still gives a strong purple color when boiled with Millon's reagent. Free di-iodotyrosine (Vaubel, 1900) and, as Harrington and Neuberger (1936) have shown, insulin whose tyrosine groups have been iodinated do not give a color with Millon's reagent.

Since submitting this paper I have found conditions under which the absorption of only 1 cc. of 0.001 *N* iodine by 10 mg. of native egg albumin brings about the abolition of the nitroprusside test in guanidine hydrochloride solution.

If in the reaction between native egg albumin and iodine, the concentration of iodine and the time and temperature of the reaction are high enough, then all the SH and S-S groups are oxidized beyond the S-S stage, as shown by a negative cyanide-nitroprusside test, and all the tyrosine groups are converted into di-iodotyrosine groups, as shown by a negative Millon test. The conditions for abolishing the cyanide-nitroprusside test are roughly the same as those for abolishing the Millon test.

I have not done any experiments to find out whether the iodine added to

native egg albumin reacts with any groups other than the SH and tyrosine groups.

Egg albumin whose SH groups have been abolished by iodine does not abolish the SH groups of untreated egg albumin in neutral guanidine hydrochloride solution.

Reactions of p-Chloromercuribenzoate with Cysteine and Native Egg Albumin.—In this section it will be shown that mercuribenzoate combines with native egg albumin either not at all or at least much more loosely than it combines with cysteine or with the cysteine in denatured egg albumin.

I have found that the compound between cysteine and mercuribenzoate, like the compound between cysteine and aldehyde (Schubert, 1936) and the cysteine in native egg albumin,² does not give a nitroprusside test or reduce ferricyanide but does reduce iodine. Thus the nitroprusside and ferricyanide tests cannot be used to find out whether mercuribenzoate has combined with the SH groups of native egg albumin because these groups do not react with nitroprusside and ferricyanide even when they are not combined with mercuribenzoate. On the other hand, the iodine reaction cannot be used either because SH reduces iodine even when it is combined with mercuribenzoate. I have accordingly used an indirect procedure involving the addition of free cysteine. If mercuribenzoate added to egg albumin is tightly bound to the protein, it cannot combine with added cysteine and the added cysteine is then free to reduce ferricyanide.

If first 1 cc. of 0.001 M cysteine and then 1 cc. of 0.001 M ferricyanide are added to 10 mg. of either native egg albumin or to denatured egg albumin in Duponol PC solution, the ferricyanide is reduced by the cysteine. In the absence of cysteine, native egg albumin does not reduce ferricyanide under any conditions and denatured egg albumin does not reduce ferricyanide under the conditions used, namely, low temperature, dilute ferricyanide, and short time of reaction.

If 1 cc. of 0.001 M mercuribenzoate is added to the *native* albumin before the addition of cysteine and ferricyanide, the ferricyanide is not reduced. This shows that the cysteine has combined with the mercuribenzoate. Either the mercuribenzoate does not combine with the native protein or it is rapidly withdrawn from its combination by the addition of cysteine. In contrast, if the mercuribenzoate is added to *denatured* egg albumin before the addition of cysteine and ferricyanide, the ferricyanide is reduced. Mercuribenzoate remains attached to the SH groups of denatured egg albumin, for a short time at least, even if cysteine is added.

SH Groups and Protein Structure.—The present results and indeed all the work on the SH groups of egg albumin and other proteins show that a

² I do not mean to suggest that the SH in native egg albumin is linked to aldehyde or heavy metal or in any other way. It seems to me more likely on the basis of the present inconclusive evidence that the SH groups of native egg albumin are not linked.

reagent which reacts with the SH groups of free cysteine may or may not react with cysteine bound in a protein. Whether or not the reaction takes place depends on what SH reagents and proteins are used, on the concentration of these substances and the time, temperature, and pH of the reaction, on whether the protein is native or denatured, on whether the solution of denatured protein contains substances such as guanidine hydrochloride or Duponol PC, and on whether the solution contains catalysts such as zinc and copper salts or inhibitors such as cyanide.

It would, of course, be desirable to be able to explain the now rather extensive experimental results in terms of some theory of protein structure. The facts, however, although they lead to vague general conclusions about the structural changes involved in denaturation, do not as yet provide proof of any definite, detailed picture of the structural relationships of SH groups in native and denatured proteins. *A priori*, a protein SH group may fail to react with an SH reagent because the protein SH group is inaccessible, or bound, or made unreactive by neighboring protein structure. *A priori*, several of these factors may operate at once, or one factor may be decisive under one set of conditions, another factor decisive under a different set of conditions. As more facts accumulate the arbitrary assumptions which can be made in connection with the three kinds of structural theories become more and more restricted.

It should be remembered that in some proteins S-S (Walker, 1925) and tyrosine (Mirsky and Anson, 1936) groups which are not detectable in the native protein are detectable by the same tests in the denatured form of the protein. The problem of how denaturation and other changes in protein structure produce changes in the properties of protein groups is not peculiar to SH groups.

It should also be remembered that the SH groups of cysteine are more readily oxidized in neutral than in acid solution and that the SH groups of cysteine are more readily oxidized than the SH groups of glutathione (Anson, 1939). Thus molecular structure can greatly influence the properties of SH groups even in relatively simple SH compounds.

EXPERIMENTAL

Reagents.—Egg albumin is thrice recrystallized with ammonium sulfate, dialyzed, and stored frozen.

Duponol PC (Du Pont) is stored at room temperature as a filtered 10 per cent stock solution.

A 5 per cent solution of ground sodium nitroprusside is made fresh daily and stored in ice water. Nitroprusside dissolves slowly unless it is first ground.

The phosphate buffer consists of equal parts of 1.0 M Na_2HPO_4 and NaH_2PO_4 .

For the ferricyanide titration reagent grade potassium ferricyanide is used. When the amount of ferrocyanide formed from ferricyanide is measured either a correction is made for the ferrocyanide present in commercial ferricyanide or the ferrocyanide is removed by oxidation with bromine (Anson, 1939). Bromine in addition to oxidizing ferrocyanide to ferricyanide also brings about some other reactions which result in darkening of the solution. This darkening is greater in the original procedure in which the bromine is added step-wise than in the following simpler procedure in which the bromine is added in dilute solution all at once, and in excess. 0.6 M ferricyanide is made up and centrifuged to remove insoluble matter. The ferrocyanide impurity is estimated by adding to 2 cc. ferricyanide solution 7 cc. of water, 0.5 cc. of 2 N sulfuric acid, and 0.5 cc. of ferric sulfate solution. The amount of red light absorbed by the Prussian blue formed is compared with the red light absorbed by a known amount of ferrocyanide in the absence of ferricyanide. Saturated bromine water is diluted 75 times with water and assumed to be 0.005 N which allows for a 10 per cent loss on dilution. If it is desired to know the concentration of bromine in the dilute solution accurately, an excess of iodide is added to a sample and the iodine liberated by bromine is titrated with thiosulfate. Twice the amount of bromine theoretically needed to oxidize the ferrocyanide present is added to the ferricyanide solution and water is added to make the ferricyanide 0.4 M. After the solution has stood 20 minutes the excess bromine is removed by aeration and the solution is allowed to stand overnight before being used. The purified ferricyanide is stored in the cold in a dark bottle. Since in the course of months ferrocyanide forms in the ferricyanide solution the ferricyanide solution is occasionally tested for ferrocyanide with ferric sulfate.

Ferric sulfate containing gum ghatti is prepared according to Folin and Malmros (1929).

A stock 0.1 M thiosulfate solution containing 0.1 gm. sodium carbonate per liter is standardized with the iodine formed by the reaction between iodide and iodate (Peters and Van Slyke, 1932).

The stock 0.1 iodine solution contains 0.18 N KI. The stock 0.1 N iodine previously used (Anson, 1940a) contained only 0.12 N KI and lost some iodine on dilution with water. The iodine solution is first made up roughly and then titrated with thiosulfate.

Tetrathionate is formed by adding just enough thiosulfate to iodine to abolish the starch test. It is made up just before being used.

p-chloromercuribenzoic acid is prepared according to the directions of Whitmore and Woodward (1932) and dissolved as the sodium salt. The solution is stable for a few days at least if stored frozen. I am indebted to Dr. Leslie Hellerman for the mercuribenzoic acid.

The guanidine hydrochloride used in the present experiments was a satisfactory batch of the Eastman product. Since guanidine hydrochloride is very hygroscopic it is first dried in a desiccator, then distributed in a number of small containers which are tightly stoppered, and stored in the cold or in some dry atmosphere.

Most samples of the commercial guanidine hydrochloride have not proved satisfactory. Good guanidine hydrochloride should dissolve in an equal weight of water to give a clear and colorless solution. The color given with egg albumin and nitroprusside in guanidine hydrochloride solution should be the same as when guanidine hydrochloride recrystallized from water is used and should not be increased if 1 drop of 0.1 N cyanide is present. The rate of fading of the color should not be much greater than that obtained

with recrystallized guanidine hydrochloride. Letting the albumin stand in neutral guanidine hydrochloride solution for 30 minutes before the addition of nitroprusside should not decrease the color obtained when nitroprusside is added. The same amount of ferricyanide should be required to abolish the nitroprusside test of denatured egg albumin in guanidine hydrochloride solution whether the ferricyanide is added before or 30 minutes after the addition of guanidine hydrochloride. Details of the nitroprusside test and the ferricyanide titration are given in later sections.

Guanidine hydrochloride can be recrystallized from water with a yield of 21 per cent. This recrystallization is used to obtain a good product for comparative purposes, not to prepare the bulk of guanidine hydrochloride used. 20 gm. of guanidine hydrochloride are dissolved in 16 cc. of water at 50°C. The solution is cooled in salt ice water and the guanidine hydrochloride is filtered off in the cold on a pre-cooled Buchner funnel. After the filter cake has been sucked and pressed as dry as possible it is placed in a desiccator.

As much as 5 gm. of guanidine hydrochloride can be dissolved in 1 cc. of water at 100°C. On cooling, however, too thick a suspension of solid is obtained for purification purposes.

Recrystallization from 80 per cent alcohol gives a 55 per cent yield and always improves the product. A completely satisfactory product is obtained from a single recrystallization, however, only if the original amount of impurities is not too great. 20 gm. of guanidine hydrochloride are dissolved in 11.4 cc. of 80 per cent alcohol at 100°C. The solution is brought about as quickly as possible, since guanidine hydrochloride gradually changes into a water-insoluble material at high temperatures. The solution is cooled and filtered as before, washed with cold absolute alcohol, and dried in a vacuum desiccator over NaOH.

Satisfactory guanidine hydrochloride can be prepared from purified guanidine carbonate. Guanidine carbonate is not hygroscopic and it is much less soluble than the hydrochloride.

First, guanidine carbonate (American Cyanamid) is stirred up with twice its weight of water and filtered. If the first part of the filtrate is not perfectly clear it is refiltered. An equal volume of 95 per cent alcohol is added to the filtrate with mechanical stirring. The resulting suspension is cooled to 0°C., filtered in the cold on a Buchner funnel, washed with cold 95 per cent alcohol, and sucked and pressed as dry as possible. Concentrated hydrochloric acid is added to the solid carbonate first with hand stirring and when the suspension becomes fluid with mechanical stirring. When the fizzing on the addition of a drop of acid becomes weak, 1.0 N hydrochloric acid is added to complete the neutralization to green to brom thymol blue. 1 or 2 cc. of water are added to a drop of guanidine hydrochloride solution before the indicator test. The solution should remain green to the indicator even after continued stirring since the carbon dioxide formed is not removed immediately. The solution is allowed to stand in the cold for a few hours, filtered to remove a small amount of brown precipitate, kept at 50°C. in a vacuum oven for 24 hours, and finally dried completely in a vacuum desiccator. During the drying the material is stirred occasionally to break up the caking.

Since guanidine carbonate is not stable indefinitely at 50°C. if the amount of solution being dried is too great to be handled by the vacuum oven in 24 hours, either the solution is evaporated in successive small portions or the solution is first evaporated to a thick suspension on an electric hot plate. The solution heated on the hot plate is placed in a Pyrex Top of the Oven frying pan, is stirred with an L shaped glass stirrer, and a blast

of air from a strong fan is directed on the solution. Under these conditions rapid evaporation takes place without the temperature going above 50°C., or during most of the evaporation above 37°C. This procedure is extraordinarily simple and effective.

The Nitroprusside Test.—The nitroprusside test in guanidine hydrochloride solution is carried out as follows. To 0.5 cc. of 1 or 2 per cent egg albumin there are added 2 drops of neutral 1.0 M phosphate, and 0.7 gm. guanidine hydrochloride. The tube containing the solution is placed in 37°C. water for 2-3 minutes and then in ice water. After the solution has been cooled, there are added 1 drop of 5 per cent sodium nitroprusside and 1 drop of 27 per cent ammonia. 0.5 cc. of 2 per cent egg albumin gives, within a third, as strong a color as 0.5 cc. of 0.002 M cysteine. The conditions for a quantitative nitroprusside test have not been worked out.

A little cyanide can be added to combine with traces of heavy metal compounds. 1 drop of 0.1 N KCN or NaCN is added before the addition of guanidine. No nitroprusside test is obtained in the presence of this small amount of cyanide with cystine or egg albumin whose SH groups have been oxidized to S-S groups by the addition of ferricyanide in guanidine hydrochloride solution, as described in a later section.

The S-S form of egg albumin gives a nitroprusside test when strong cyanide is added which can reduce S-S to SH. To a guanidine hydrochloride solution of the S-S protein, 1 drop of 2 N NaCN and 1 drop of ammonia are added, the solution is allowed to stand 5 minutes at room temperature and is then cooled in ice water. On the addition of 1 drop of nitroprusside a pink color is obtained.

If the nitroprusside test described in the first paragraph of this section is carried out in a solution of denatured egg albumin containing urea instead of guanidine hydrochloride the color obtained is much less intense than if guanidine hydrochloride is used.

If egg albumin is denatured by trichloroacetic acid or Duponol PC and no guanidine hydrochloride or urea is present then only a negligible weak pink color is obtained on the addition of nitroprusside and ammonia. If the ammoniacal solution of egg albumin denatured by trichloroacetic acid or Duponol PC is saturated with ammonium sulfate, the protein is precipitated and this precipitate gives a faint pink color with nitroprusside. This faint pink color is much intensified on the further addition of solid guanidine hydrochloride or solid thiocyanate.

Titrations in Guanidine Hydrochloride Solution.—First, the standard titration procedure. To 0.5 cc. of 2 per cent egg albumin there are added 0.1 cc. of buffer solution containing equal parts of 1.0 M Na_2HPO_4 and 1.0 M NaH_2PO_4 , 0.5 cc. of 0.002 M ferricyanide, tetrathionate, or mercuribenzoate, and 1.2 gm. of guanidine hydrochloride of tested purity. The solution is placed in 37°C. water for 3 minutes and cooled in ice water. Then 1 drop of 5 per cent nitroprusside and 1 drop of 27 per cent ammonia are added. No pink color is observed. If 1 drop of 0.1 N NaCN is added before the nitroprusside, still no pink color is obtained. If 0.5 cc. of 0.0018 M ferricyanide, tetrathionate, or mercuribenzoate is added, then a weak pink is obtained about equal to that obtained from 0.5 cc. of 0.2 per cent albumin to which no SH reagent has been added.

If 0.5 cc. of 0.002 M ferricyanide or mercuribenzoate is used, no nitroprusside test is obtained whether the nitroprusside test is carried out as quickly as possible after the solution of the guanidine and cooling of the solution or after the solution containing protein, ferricyanide, and guanidine has stood 30 minutes at 37°C. If 0.5 cc. of 0.0018 M ferricyanide or mercuribenzoate is used a small nitroprusside test is obtained whether the test is carried out as soon as possible or after 30 minutes.

If the guanidine hydrochloride used is free of impurities which bring about the abolition of SH groups, the ferricyanide can be added 30 minutes after the guanidine hydrochloride without any change in the amount of ferricyanide needed to abolish the nitroprusside test. To 0.5 cc. of protein solution plus 0.1 cc. of phosphate buffer there is added 0.7 gm. of guanidine hydrochloride. The solution is allowed to stand 30 minutes at 37°C., 0.5 cc. of ferricyanide is added, the solution is allowed to stand 3 minutes more at 37°C. before being cooled in ice water. Finally 1 drop of nitroprusside and 1 drop of ammonia are added to find out whether the SH groups have all been oxidized.

The standard titration can be carried out with 0.5 cc. of 0.4 per cent egg albumin instead of 0.5 cc. of 2 per cent egg albumin. The less protein is used, the weaker the nitroprusside test given when only 10 per cent of the SH groups survive. Instead of titrating a dilute solution, one can concentrate the protein. A volume of egg albumin solution containing 10 mg. of protein is diluted to 9 cc. with water and 1 cc. of 2.0 N trichloroacetic acid is added. The precipitate is centrifuged down and dissolved with the minimum amount of 0.5 N sodium hydroxide and the resulting solution is diluted to approximately 0.5 cc. with water. Then buffer, titrating agent, and guanidine hydrochloride are added as in the standard procedure.

Formaldehyde cannot be used as a titrating agent because it abolishes the protein SH groups only when added in excess. If 0.5 cc. of 0.004 M formaldehyde is added to the 0.5 cc. of 2 per cent egg albumin before the guanidine under the standard titration conditions, a strong positive nitroprusside is obtained. If 0.5 cc. of 38 per cent formaldehyde is added, only a slight flash of pink is observed.

Effect of Cyanide on Titrations.—The ferricyanide titration cannot be carried out in the presence of cyanide because cyanide in some way inhibits the reduction of ferricyanide by denatured egg albumin, as shown by the following experiment. 1 drop of 0.1 N NaCN is added to the protein solution before the addition of phosphate, ferricyanide, and guanidine. A strong nitroprusside test is obtained although in the absence of cyanide the nitroprusside test would be negative.

Although concentrated ferricyanide oxidizes cyanide slowly, under the conditions of the experiments just described no ferrocyanide is formed from ferricyanide by a cyanide-guanidine hydrochloride-phosphate solution which does not contain protein.

0.5 cc. of 0.002 M free cysteine in a guanidine hydrochloride-phosphate solution reduces ferricyanide in the presence as well as in the absence of 1 drop of 0.1 N NaCN. In the presence of the cyanide, however, the disappearance of the brown ferricyanide color is slow enough to be observed. In the absence of cyanide the disappearance of the brown color takes place instantaneously so far as the eye can tell.

Just as 10 mg. of denatured egg albumin in guanidine hydrochloride solution still gives a nitroprusside test after being treated with 0.5 cc. of 0.002 M ferricyanide in the presence of 1 drop of 0.1 N cyanide, so a strong nitroprusside is also obtained after treatment of 10 mg. denatured egg albumin with 0.5 cc. of 0.002 M mercuribenzoate in the presence of cyanide. Presumably cyanide combines with the heavy metal in mercuribenzoate and so prevents the mercuribenzoate from combining with SH groups. In contrast, 0.5 cc. of 0.002 M tetrathionate abolishes the nitroprusside test in the presence as well as in the absence of 1 drop of 0.1 N cyanide.

Tests for Completeness of SH Reactions in Neutral Guanidine Hydrochloride Solution.—The following series of experiments was designed to find out whether the abolition of the SH groups by the SH reagents takes place entirely in the 3 minute reaction in neutral

solution, or whether part of the abolition of SH groups takes place after ammonia is added for the nitroprusside test.

After the protein and titrating agent have been in the neutral guanidine hydrochloride solution for 3 minutes at 37°C. under the conditions of the standard titration water is added to 9 cc. and then 1 cc. of 2.0 N trichloroacetic acid. The precipitate is centrifuged, washed with 0.2 N trichloroacetic acid, and centrifuged again. Water is added to the precipitate to make the volume approximately 1 cc. (previously marked on the tube) and the precipitate is dissolved with 1 gm. of guanidine hydrochloride and cooled in ice water. Then nitroprusside and ammonia are added. The protein treated with ferricyanide for 3 minutes gives no nitroprusside test, the protein treated with tetrathionate a moderately strong test, the protein treated with mercuribenzoate about as strong a test as untreated protein. The experiments are repeated, trichloroacetic being added after tetrathionate and mercuribenzoate have stood in the neutral protein-guanidine solution for 30 minutes instead of 3 minutes. This time the protein treated with tetrathionate gives no nitroprusside test (even if 1 drop of 0.1 N cyanide is present during the tetrathionate reaction) but the protein treated with mercuribenzoate still gives as strong a test as before. Thus the reaction between ferricyanide and the SH groups of denatured egg albumin in neutral guanidine solution (before the addition of ammonia) is completed in 3 minutes, whereas the reaction with tetrathionate is completed in 30 minutes but not in 3 minutes.

The fact that the trichloroacetic acid precipitate of albumin treated with mercuribenzoate gives a nitroprusside test shows that the compound between mercuribenzoate and the protein SH groups must be dissociated by trichloroacetic acid, for the following experiment shows that mercuribenzoate actually does combine with the SH groups of denatured egg albumin in neutral solution. After the protein solution containing guanidine hydrochloride and mercuribenzoate has stood 3 minutes, 2 drops of 0.01 M ferricyanide are added. The protein after being precipitated and washed with trichloroacetic acid still gives a strong nitroprusside test in guanidine solution. If mercuribenzoate is omitted the nitroprusside test is abolished by ferricyanide. Thus mercuribenzoate prevents the oxidation of the SH groups by ferricyanide in neutral guanidine solution.

Ferricyanide Reduction Test for SH Groups.—The SH groups of the trichloroacetic acid precipitate of protein treated with ferricyanide or tetrathionate in guanidine hydrochloride solution can also be measured by the ferricyanide-Duponal PC method. The results confirm those obtained by the nitroprusside test.

The washed trichloroacetic acid precipitate is dissolved by the addition of 0.5 cc. of 10 per cent Duponal PC, 0.3 cc. of 0.5 N NaOH (to neutralize the trichloroacetic acid), and 0.2 cc. of the neutral phosphate buffer. 0.5 cc. of 0.1 M ferricyanide is added and after the solution has been in a 37°C. bath for 10 minutes the ferrocyanide formed is measured as Prussian blue as described in the following section. The proteins treated with ferricyanide for 3 minutes or with tetrathionate for 30 minutes give no ferrocyanide just as they give no nitroprusside test. The protein treated with tetrathionate for 3 minutes gives the equivalent of 1 cc. of 0.0006 M ferrocyanide just as it gives a moderate nitroprusside test. Protein originally treated with 1 cc. of 0.0008 M ferricyanide gives 1 cc. of 0.00016 M ferrocyanide just as it gives a small nitroprusside test.

The procedure just described can be used to test the effect of cyanide on the oxidation of the SH groups of egg albumin by ferricyanide and tetrathionate. 10 mg. of denatured egg albumin in guanidine hydrochloride solution are exposed for 30 minutes to 0.5 cc.

of 0.002 M ferricyanide or tetrathionate in the presence of 1 drop of 0.1 cyanide. The protein is then precipitated and washed with trichloroacetic acid, dissolved in neutral Duponol PC solution, and the surviving SH groups measured with ferricyanide. Although in the absence of cyanide all the SH groups are abolished, in the presence of cyanide ferricyanide abolishes only 55 per cent of the SH groups, tetrathionate 95 per cent. This confirms the result obtained by the nitroprusside test that cyanide interferes with the ferricyanide reaction more than it interferes with the tetrathionate reaction.

Ferricyanide Reduction in Guanidine Hydrochloride Solution.—To 0.5 cc. of 5 per cent egg albumin there are added 2 drops of phosphate buffer, 1 drop of 0.1 or 0.5 M ferricyanide, and 0.6 gm. guanidine hydrochloride. After the solution has been kept in a 37°C. water bath for 3 minutes there are added 1 cc. of water, 0.5 cc. of 2.0 N sulfuric acid, 18 cc. of water, and 2.5 cc. of 2.0 N trichloroacetic acid. The suspension is well mixed and centrifuged. If any particles remain in the supernatant solution, the centrifuging is repeated. Filtration results in some loss of ferrocyanide. To 9 cc. of the supernatant solution there are added 0.5 cc. of 0.1 M ferricyanide and 0.5 cc. of ferric sulfate solution. After 20 minutes the Prussian blue is read in the light transmitted by a red filter against the Prussian blue developed from 1 cc. of 0.0025 M ferrocyanide. The two color values agree within 5 per cent. The ferrocyanide standard is made up as follows. To 0.5 cc. of 5 per cent egg albumin there are added 2 drops phosphate solution and 0.6 gm. guanidine hydrochloride. After the solution has stood at 37°C. for 3 minutes 1 cc. of 0.0025 M ferrocyanide is added. Then sulfuric acid and the other reagents are added as before to develop the Prussian blue.

Since guanidine hydrochloride in sufficient concentration interferes with the development of Prussian blue, the experiment is arranged so as to keep the concentration of guanidine hydrochloride as low as possible.

Guanidine hydrochloride, unlike Duponol PC, does not prevent the precipitation of denatured egg albumin by acid ferric sulfate. That is why the protein is precipitated with trichloroacetic acid and removed before the addition of ferric sulfate. Duponol PC cannot be added to the acid solution to keep the protein in solution because it forms a precipitate with guanidine hydrochloride.

Ferricyanide Reduction in Urea Solution.—In one test tube there are added to 0.5 cc. of 2 per cent egg albumin 2 drops of 1 N hydrochloric acid and 0.6 gm. urea. After this test tube has been at 37°C. for 5 minutes there is added from another test tube a mixture of 0.5 cc. 0.002 M or 0.1 M ferricyanide, 2 drops 1 N sodium hydroxide, 4 drops 1 M neutral phosphate, and 0.8 gm. urea. After 5 minutes more at 37°C. the reaction is stopped by 0.5 cc. of 2 N sulfuric acid and then there are added 0.5 cc. of 0.1 M ferricyanide (only to the solution containing 0.5 cc. of 0.002 M ferricyanide), water to 9.5 cc., and 0.5 cc. ferric sulfate solution. The Prussian blue formed is equivalent to 1 cc. of 0.001 M ferrocyanide, within 5 per cent, whether 0.002 M or 0.1 M ferricyanide is originally added.

Titrations in Duponol PC Solution.—First the mercuribenzoate titration. To 1 cc. of 1 per cent egg albumin are added 2.3 cc. of water, 0.2 cc. of the neutral 1.0 M phosphate, and 0.5 cc. of 10 per cent Duponol PC. The solution is brought to 37°C. and 0.5 cc. of 0.02 M ferricyanide is added. After the solution has been at 37°C. for 1 minute there are added 0.5 N of 2.0 N sulfuric acid, water to 9.5 cc., and 0.5 cc. of ferric sulfate solution. After 20 minutes the Prussian blue developed is estimated colorimetrically in the light transmitted by a red filter. The Prussian blue is the same, within 5 per cent, as that developed from 1 cc. of 0.001 M ferrocyanide (*cf.* Anson, 1939). If 1 cc. of 0.001 M

mercuribenzoate is added to the protein-Duponol solution before the ferricyanide and the solution is allowed to stand 1 minute at 37°C. before the addition of ferricyanide, then no Prussian blue is obtained. Thus mercuribenzoate prevents the reduction of ferricyanide by denatured egg albumin in Duponol PC as in guanidine hydrochloride solution. In practice, to avoid the difficulty of estimating very weak colors, 1 cc. of 0.001 M ferrocyanide is added after the reaction has been stopped with sulfuric acid. The Prussian blue obtained is that expected from 1 cc. of 0.001 M ferrocyanide. If 1 cc. of 0.0008 M mercuribenzoate is used instead of 0.001 M mercuribenzoate, then on the addition of 1 cc. of 0.001 M ferrocyanide as much Prussian blue is developed (to quote a single experiment) as would be developed from 1 cc. of 0.00115 M ferrocyanide. The 0.0008 M mercuribenzoate does not completely abolish the SH groups and so some ferricyanide is reduced.

A control experiment is carried out to show that under the conditions used mercuribenzoate does not interfere with the estimation of ferrocyanide. 1 cc. of 0.001 M ferrocyanide is added after the mercuribenzoate and the ferricyanide is added after the sulfuric acid which stops all reduction of ferricyanide. The Prussian blue formed is that expected from the amount of ferrocyanide added. If, however, the solution containing mercuribenzoate and ferrocyanide is allowed to stand 5 minutes (instead of 1 minute as in the actual experiment) before the addition of acid ferric sulfate, then less than the expected amount of Prussian blue is obtained. Presumably in the presence of mercury salt and air some ferrocyanide is oxidized.

Another control experiment shows that under the conditions used mercuribenzoate does not reduce ferricyanide. After the ferricyanide has been allowed to react with denatured egg albumin in the absence of mercuribenzoate, 1 cc. of 0.001 M mercuribenzoate is added and the solution is allowed to stand 1 minute before the addition of acid and ferric sulfate. The amount of Prussian blue obtained is the same as that obtained when mercuribenzoate is not added.

For the ferricyanide and tetrathionate titrations in Duponol PC solution the nitroprusside test is used for the end point. To 0.5 cc. of 2 per cent egg albumin are added 0.2 cc. phosphate solution, 0.5 cc. of 0.002 M ferricyanide or tetrathionate, and 0.5 cc. of 0.8 per cent Duponol PC solution. After this solution has stood 10 minutes in the ferricyanide titration and 30 minutes in the tetrathionate titration water is added to 9 cc., the protein is precipitated by the addition of 1 cc. 2.0 N trichloroacetic acid and warming of the solution to 60°C., washed with 0.2 N trichloroacetic acid, diluted to 1 cc., and dissolved with 1 gm. of guanidine hydrochloride. No pink color is obtained on the addition of nitroprusside and ammonia. If 10 per cent less ferricyanide or tetrathionate is used, a weak color is obtained in the nitroprusside test.

Only 4 mg. of Duponol PC is used in the experiment just described because larger amounts of Duponol interfere with the precipitation of the protein with trichloroacetic acid. 10 mg. of Duponol PC does not prevent the precipitation of the ordinary SH form of egg albumin, but it prevents the precipitation by trichloroacetic acid if the protein SH groups are first oxidized to S-S groups.

A control experiment shows that even when only 4 mg. of Duponol PC is used to denature the egg albumin, all the 0.5 cc. of 0.002 M ferricyanide added is reduced to ferrocyanide. After the ferricyanide has reacted with the denatured egg albumin in neutral Duponol solution, there are added 0.5 cc. of 2.0 N sulfuric acid, 0.5 cc. of 10 per cent Duponol PC, 0.5 cc. of 0.1 M ferricyanide, water to 9.5 cc., and 0.5 cc. of the ferric

sulfate solution. As much Prussian blue is formed, within 5 per cent, as from 1 cc. of 0.001 M ferrocyanide. Extra Duponol is added after the reaction has been stopped by sulfuric acid to prevent the precipitation of protein by acid ferric sulfate. Extra ferricyanide is added to speed up the formation of Prussian blue in Duponol solution.

Cyanide inhibits the reduction of ferricyanide by denatured egg albumin in Duponol solution even more than it does in guanidine hydrochloride solution. If in the experiment just described 1 drop of 0.1 N cyanide is added before the addition of Duponol and the ferricyanide is in contact with the albumin 30 minutes, only 1 cc. of 0.00007 M ferrocyanide is formed. In contrast 0.5 cc. of 0.002 M tetrathionate abolishes the nitroprusside test of 10 mg. of denatured albumin even if 1 drop of 0.1 N cyanide is present during the tetrathionate reaction.

Reactions of Iodine with Native Egg Albumin.—First, the experiments showing the minimum amount of iodine which abolishes the nitroprusside test. To 0.5 cc. of 2 per cent egg albumin are added at 0°C. 0.1 cc. of neutral 1.0 M phosphate and 0.5 cc. of 0.0026 N iodine. The solution is allowed to stand 5 minutes at 0°C., during which time all the iodine added is absorbed as shown by a negative starch test. The egg albumin treated with iodine whether dialyzed free of iodide or not gives a negative nitroprusside test in guanidine hydrochloride solution but if it is first allowed to stand in an alkaline solution containing strong cyanide, a strong positive nitroprusside test is obtained. The techniques of the nitroprusside tests are described in the section on the nitroprusside test. If 10 per cent less iodine is used a weak positive nitroprusside test is obtained without the preliminary treatment with alkaline cyanide.

In the next experiments more iodine is added at 37°C., and there are no surviving S-S groups which give the nitroprusside test after exposure to alkaline cyanide and no uniodinated tyrosine groups which give the Millon test. To 0.5 cc. of 2 per cent egg albumin there are added 0.1 cc. of phosphate buffer and 0.5 cc. of 0.08 N iodine. The solution is allowed to stand 2 hours at 37°C. Then water is added to 9 cc. and 1 cc. of 2.0 N trichloroacetic acid. The precipitate is centrifuged, stirred up with 10 cc. of 0.2 N trichloroacetic acid, and centrifuged again. The precipitate is suspended in enough water to make the volume approximately 1 cc., and is dissolved with 1.2 gm. guanidine hydrochloride, and 1 drop of 2.0 N cyanide and 1 drop of ammonia are added. After 5 minutes 1 drop of 5 per cent nitroprusside is added. No pink color is obtained. A Millon test is carried out on the 0.5 cc. of the precipitate washed with trichloroacetic acid by adding 3 drops of Millon's reagent and heating in boiling water for 2-3 minutes. No purple color is obtained. The Millon reagent used is made up as follows. 10 gm. of mercury are digested in 20 gm. of nitric acid of specific gravity 1.42 until NO_2 no longer comes off. The solution is diluted with twice its volume of water and stored in a brown bottle. In carrying out the Millon test it is necessary to heat long enough to bring out the full color and not long enough to make the color disappear again.

If 0.06 N iodine is added to 37°C. in the experiment just described, instead of 0.08 N iodine, the protein after being precipitated with trichloroacetic acid gives a weak cyanide-nitroprusside test and a weak Millon test.

If after the iodine is added the solution is allowed to stand 1 hour at 60°C. instead of 2 hours at 37°C., 0.5 cc. of 0.05 N iodine has to be added to abolish the cyanide-nitroprusside and the Millon test of 0.5 cc. of 2 per cent egg albumin. If 0.04 N iodine is added faint positive tests are obtained.

A control experiment is done to show that the trichloroacetic precipitation and washing adequately removes the tetrathionate formed by the reaction of iodine and thiosulfate.

0.5 cc. of 0.08 N iodine and 0.5 cc. 0.08 M thiosulfate are mixed before being added to egg albumin. The protein after being precipitated and washed with trichloroacetic acid gives strongly positive cyanide-nitroprusside and a strongly positive Millon test.

Mercuribenzoate Plus Native and Denatured Egg Albumin.—The following experiments show that if mercuribenzoate combines with native egg albumin at all, the compound is much looser than the compound between mercuribenzoate and denatured egg albumin.

First it is shown that under the conditions used ferricyanide is not reduced by either native egg albumin or by denatured egg albumin in Duponol solution. To 1 cc. of 1 per cent native egg albumin are added 0.2 cc. of phosphate buffer and 0.5 cc. of water. The solution is cooled to 0°C. 1 cc. of 0.001 M ferricyanide previously cooled to 0°C. is added, the solution is allowed to stand 1 minute at 0°C., and then there are added 0.5 cc. of 2.0 N sulfuric acid, 1 cc. of 0.001 M ferrocyanide, 0.5 cc. of 0.02 M ferricyanide, 0.5 cc. of 10 per cent Duponol PC, water to 9.5 cc., and 0.5 cc. ferric sulfate solution. The Prussian blue obtained is the same in amount as the Prussian blue obtained from 1 cc. of 0.001 M ferrocyanide alone, indicating no reduction of ferricyanide by native egg albumin.

The same experiment is repeated with denatured egg albumin, 0.5 cc. of 10 per cent Duponol PC being added to 1 cc. of egg albumin instead of 0.5 cc. of water and no Duponol being added after the acid. Again no ferricyanide is reduced.

The next experiments show that cysteine added to native or denatured egg albumin is free to reduce ferricyanide. 1 cc. of cold 0.001 N cysteine (in 0.01 N hydrochloric acid) is added to the cold native or denatured egg albumin, the ferricyanide added as promptly as possible after the cysteine, and the acid is added 1 minute later. The equivalent of 0.75 – 0.9 cc. of ferrocyanide is formed. Some cysteine is unavoidably oxidized by the oxygen of the air and the amount oxidized is variable.

Finally, the experiments which show whether or not added mercuribenzoate is free to combine with cysteine. 1 cc. of 0.001 M mercuribenzoate is added to the native or denatured egg albumin before the solution is cooled and cysteine and ferricyanide are added. In the solution of native egg albumin no ferrocyanide is formed, showing that the mercuribenzoate has combined with the cysteine and that the cysteine-mercuribenzoate compound does not reduce ferricyanide. In the solution of denatured egg albumin, however, the same amount of ferrocyanide is formed from ferricyanide as in the absence of mercuribenzoate, showing that the mercuribenzoate has combined with the protein and is not removed from the protein by the cysteine which remains free to reduce ferricyanide.

If the experiment in Duponol solution is repeated with 1 cc. of water substituted for the 1 cc. of protein solution, no ferrocyanide is formed, showing that mercuribenzoate can combine with the cysteine in the Duponol solution if the mercuribenzoate is not combined with protein.

The Mercuribenzoate-Cysteine Compound.—If 1 cc. of 0.001 M cysteine is added to a neutral phosphate solution containing 1 cc. of 0.0001 M mercuribenzoate, the resulting solution does not give a nitroprusside test but does immediately decolorize 1 cc. of 0.001 N iodine solution.

SUMMARY

1. 1 cc. of 0.001 M ferricyanide, tetrathionate, or *p*-chloromercuribenzoate is required to abolish the SH groups of 10 mg. of denatured egg albumin in

guanidine hydrochloride or Duponol PC solution. Both the nitroprusside test and the ferricyanide reduction test are used to show that the SH groups have been abolished.

2. 1 cc. of 0.001 M ferrocyanide is formed when ferricyanide is added to 10 mg. of denatured egg albumin in neutral guanidine hydrochloride or urea solution. The amount of ferricyanide reduced to ferrocyanide by the SH groups of the denatured egg albumin is, within wide limits, independent of the ferricyanide concentration.

3. Ferricyanide and *p*-chloromercuribenzoate react more rapidly than tetrathionate with the SH groups of denatured egg albumin in both guanidine hydrochloride solution and in Duponol PC solution.

4. Cyanide inhibits the oxidation of the SH groups of denatured egg albumin by ferricyanide.

5. Some samples of guanidine hydrochloride contain impurities which bring about the abolition of SH groups of denatured egg albumin and so interfere with the SH titration and the nitroprusside test. This interference can be diminished by using especially purified guanidine hydrochloride, adding the titrating agent before the protein has been allowed to stand in guanidine hydrochloride solution, and carrying out the nitroprusside test in the presence of a small amount of cyanide.

6. The SH groups of egg albumin can be abolished by reaction of the native form of the protein with iodine. It is possible to oxidize all the SH groups with iodine without oxidizing many of the SH groups beyond the S-S stage and without converting many tyrosine groups into di-iodotyrosine groups.

7. *p*-chloromercuribenzoate combines with native egg albumin either not at all or much more loosely than it combines with the SH groups of denatured egg albumin or of cysteine.

8. The compound of mercuribenzoate and SH, like the compound of aldehyde and SH and like the SH in native egg albumin, does not give a nitroprusside test or reduce ferricyanide but does reduce iodine.

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REACTION OF FOLIN'S REAGENT WITH PROTEINS AND BIURET COMPOUNDS IN PRESENCE OF CUPRIC ION

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In an earlier paper¹ the writer noted that traces of copper sulfate increased the blue color obtained by treating such proteins as pepsin or gelatin with Folin's phenol reagent.

It has now been found that a number of organic substances reduce the Folin's phenol reagent only if a small amount of cupric ion is present. These same substances also show a positive biuret reaction.

The copper-phenol values in Table I represent the amount of color per milligram of substance produced as a result of adding $m/1300$ copper sulfate to the solution under examination with the phenol reagent. One obtains this value from the difference in color values produced with and without copper ion. For many substances no color is produced in the absence of copper but most proteins contain tyrosine and tryptophane and must, therefore, be analyzed with and without cupric ion. No attempt has been made to determine whether each of the substances in Table I produces a blue color exactly in proportion to its concentration. With leucyl-glycyl-glycine the proportionality is quite good while with pure biuret it is not as good. The order of magnitude of the values in Table I is of more significance than the absolute values. The solutions have been read against a tyrosine standard and the values calculated as the number of milligrams of tyrosine that will give the same amount of color under identical conditions.

No amino acids show any comparable effect of the presence of cupric ion when tested with the phenol reagent.

With certain substances, particularly biuret, the presence of some non-chromogenic substances has fairly pronounced effect on the amount of blue color that develops in the presence of cupric ion. Thus the presence of glycine or glycyl-glycine increases the copper-phenol color of biuret. Urea gives a blue color with the phenol reagent in the presence of copper ion and will, therefore, interfere if present in a solution being analyzed.

In view of the fact that the products of hydrolysis have little if any influence on the color of leucyl-glycyl-glycine, it seems likely that one could follow

¹Herriott, R. M., *J. Gen. Physiol.*, 1935, 19, 287.

quantitatively the extent of enzymatic hydrolysis of this and similar peptides by means of this color test

The amount of copper required for maximal color development varies somewhat with different substances but it has been found that 1 ml of M/100 copper

TABLE I

| Substance | Amount used, mg | Copper-phenol color value per mg material,* mg tyrosine |
|----------------------------|-----------------|---------------------------------------------------------|
| Tyrosine | 0 15 | 0 |
| Biuret† | 0 1 | 1 3 |
| Casein (Hammarsten) | 0 84 | 13 |
| Zein | 0 81 | 17 |
| Egg albumin | 1 05 | 15 |
| Edestin | 0 74 | 23 |
| Ghadin "B" | 0 63 | 22 |
| Pepsin | 0 53 | 20 |
| Pepsin (autolysate) | 0 53 | 24 |
| Pepsin (acid hydrolysate) | 1 0 | 02 |
| Gelatin | 1 0 | 14 |
| Gelatin (acid hydrolysate) | 10 5 | 02 |
| Peptone (Roche) | 0 5 | 12 |
| Glycyl tyrosine | 0 18 | 0 |
| Glycyl-tryptophane | 0 4 | 0 |
| Glycyl leucine† | 18 9 | 005 |
| Glycyl-glycine† | 13 2 | 015 |
| Malonamide† | 1 0 | 25 |
| Oxamide† | 0 9 | 20 |
| Formamide† | 4 5 | 0 |
| Leucyl-glycyl glycine† | 0 25 | 2 |
| Histidine | 4 6 | 02 |

* This value is obtained as the difference between the value in the presence of copper ion and in the absence of it, divided by the milligrams of material used

† These substances show practically no color in the absence of copper ion

sulphate is maximal or nearly so for most of the different substances thus far used

Very little can be said about the mechanism of the reaction but it should be recalled that Wu² pointed out many years ago that as little as one part in five million of cuprous copper ion reduces the phenol reagent.

These experiments demonstrate that under certain conditions proteins or peptides which seem unreactive may, in the presence of very small amounts of a second substance, exhibit very marked reactivity.

²Wu, H., *J. Biol Chem*, 1920, **43**, 189

Experimental Details. Procedure. One ml of a solution of the substance to be studied was put into a 50 ml Erlenmeyer flask along with 1 ml of $M/100$ copper sulphate and 8 ml of 0.5 N sodium hydroxide. To this was then added dropwise with whirling 3 ml of the $\frac{1}{3}$ dilution of Folin's^a phenol reagent. The solutions were then read after 5–10 minutes against 0.15 mg tyrosine treated in a similar manner.

^aFolin, O., and Ciocalteau, V., *J. Biol. Chem.*, 1927, **73**, 627.

EFFECTS OF NITROBENZENE AND BENZENE ON VALONIA

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Nitrobenzene¹ is of special interest since, like certain living cells, it is able to discriminate electrically² between Na⁺ and K⁺.

This paper describes certain experiments in which it was applied to *Valonia*. Its effect on the P.D. is seen³ in Fig. 1. At the start the cell in sea water had a negative⁴ P.D. of 6 mv. When 0.013 M nitrobenzene in sea water was applied the P.D. after a short latent period changed in a positive direction. The curve fell and then rose very slowly: the rise is termed "recovery" for convenience, but this does not mean that the cell is returning to its normal state. A similar result is obtained with guaiacol and with hexylresorcinol and the cause may be the same in all these cases.

With guaiacol⁵ and with hexylresorcinol⁶ recovery is usually complete or nearly so but in these experiments with nitrobenzene it was very slow and often incomplete.⁷

The latent period in Fig. 1 is much shorter than with guaiacol and hexylresorcinol. But in many cases it is longer than in Fig. 1 and it may last 45 seconds. With some cells the descent of the curve is much more rapid and may resemble that found with guaiacol. The time of recovery in nitrobenzene is very variable but in no case is less than 5 minutes.

Nitrobenzene lessens the potassium effect. With normal cells replace-

¹ C₆H₅(NO₂).

² Osterhout, W. J. V., Some models of protoplasmic surfaces, in Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, Long Island Biological Association, 1940, 8, 51.

³ The experiments were made on *Valonia macrophysa*, Kütz., using the technique described in former papers (Osterhout, W. J. V., *J. Gen. Physiol.*, 1936-37, 20, 13; regarding the amplifier see Hill, S. E., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1937 38, 21, 541). The temperature varied between 20 and 25°C.

No evidence of injury was seen in these experiments.

⁴ The P.D. is called negative when the positive current tends to flow from the external solution across the protoplasm to the sap.

⁵ Cf. Osterhout, W. J. V., *J. Gen. Physiol.*, 1936-37, 20, 13.

⁶ Osterhout, W. J. V., *J. Gen. Physiol.*, 1940 41, 24, 311.

⁷ True recovery occurred when the cells were replaced in sea water.

ment of sea water by 0.6 M KCl changed the P.D. in a negative direction by 20 to 54 mv. but when both solutions contained 0.013 M nitrobenzene the change was reduced almost to zero.⁸ When sea water was replaced by "0.27 M K sea water" (sea water in which 0.27 M NaCl had been replaced by KCl) the change of P.D. in a negative direction was from 9 to 16 mv. But there was little or no change when both solutions contained 0.013 M nitrobenzene.

Evidently nitrobenzene makes K^+ and Na^+ act more nearly alike. How does this come about?

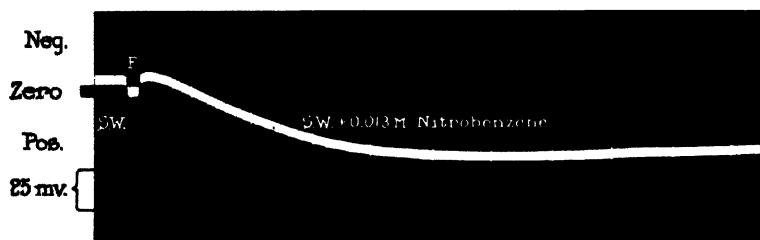


FIG. 1. At the start the cell had a negative P.D. of 6 mv. in sea water. When the cell was removed from the sea water the curve fell suddenly and registered the "free grid" (F) of the amplifier. Then nitrobenzene 0.013 M was added to the sea water and the curve jumped back to its former level and after a short latent period (including a slight rise) fell slowly until the P.D. was 41 mv. positive and later began to rise very gradually.

Temperature 24°C. Time marks 15 seconds apart.

The behavior of Na^+ is changed as shown by the dilution effect. With normal cells dilution of the sea water to one half by isotonic glycerol (1.1M) containing 0.02 M $CaCl_2$ + 0.012 M KCl changed the P.D. in a negative direction to the extent of 5 to 12 mv. This change became less or disappeared entirely when the sea water contained 0.013 M nitrobenzene.

A similar result has been obtained with guaiacol⁵ and with hexylresorcinol.⁶

This indicates that in normal cells u_{Na} is less than v_{Cl} but under the influence of the reagent this difference diminishes or disappears, making the behavior of Na^+ more like that of K^+ since normally we have $u_K > v_{Cl} > u_{Na}$.

Assuming that the partition coefficients (concentration in the non-aqueous

⁸ The cells were first tested with 0.6 M KCl and returned to sea water. After a lapse of several hours they were tested with 0.6 M KCl + 0.013 M nitrobenzene. Failure to recover signifies an altered state of the cell (when dead the P.D. is zero).

protoplasmic surface \div concentration in the external solution) are equal⁹ for KCl and NaCl it is evident that in order to abolish the potassium effect u_K must be equal to u_{Na} . Hence the loss of the potassium effect in the presence of the reagent indicates that $u_K = u_{Na} = v_{Cl}$. Since normally $u_K > v_{Cl} > u_{Na}$ this means that the reagent has increased u_{Na} and decreased u_K (assuming that v_{Cl} remains constant which is the simplest working hypothesis¹⁰).

It is of interest to note that when cells are in the "delayed polarization state"¹¹ they may be restored to the "regular polarization state" by application of nitrobenzene.

The changes in P.D. caused by nitrobenzene (Fig. 1) are antagonized to some extent by ammonia. After the positive change has occurred the addition of 0.002 M NH_4Cl at pH 8.1 usually changes the P.D. to some extent in a negative direction without necessarily bringing it back to the original value (before the nitrobenzene was added). The protoplasmic resistance¹² is increased by nitrobenzene but when ammonia is subsequently added it falls. Ammonia also tends to put the cells into the delayed polarization state.

The application of benzene (0.004 M to 0.008 M) produces effects resembling those described for nitrobenzene. Despite the differences between these substances¹³ their effects are quite similar.

⁹ The partition coefficients of KCl and of NaCl are assumed to be equal. This is done because we have no satisfactory way of estimating them. In order to make such an estimate we should need to measure the change of P.D. on diluting 0.6 M KCl (or a sea water rich in KCl) with an isotonic non-electrolyte but this is not practicable because in such solutions the P.D. constantly changes (cf. Damon, E. B., *J. Gen. Physiol.*, 1932-33, 16, 375). Hence the apparent mobility as here used includes the partition coefficient in the sense that a higher partition coefficient of KCl would make u_K appear higher. It follows that the potassium effect becomes zero only when u_K equals u_{Na} .

¹⁰ Since the resistance rises it is quite possible that v_{Cl} is not constant but it may still be true that the ratio $u_K + v_{Cl}$ diminishes and the ratio $u_{Na} + v_{Cl}$ increases which is all that the above discussion implies since when we say u_K and u_{Na} we really mean $u_K + v_{Cl}$ and $u_{Na} + v_{Cl}$, since v_{Cl} is always taken as unity. (Cf. Osterhout, W. J. V., *J. Gen. Physiol.*, 1929-30, 13, 715.)

¹¹ Blinks, L. R., *J. Gen. Physiol.*, 1935-36, 19, 633.

In this case the delayed polarization state was manifested by a lack of response or very little response to outward currents of less than 5 microamperes per square centimeter. No larger outward currents and no inward currents were applied since it seemed desirable to avoid changes in the surface due to current flow.

¹² The protoplasmic resistance was determined as described by Blinks and Skow (Blinks, L. R., and Skow, R. K., *J. Gen. Physiol.*, 1940-41, 24, 247).

¹³ The dielectric constants are, for benzene 2.2 (at 18°C.), for nitrobenzene 36 (at 20°C.). The latter is very polar as contrasted with benzene and is more soluble in water.

According to Blinks¹⁴ acids raise the resistance in *Valonia* and this is antagonized by ammonia. He suggests (personal communication) that when reagents such as nitrobenzene and benzene produce a rise in resistance which is antagonized by ammonia the change may be due to production of acid by the cell which may also cause an alteration in P.D. (in a positive or negative direction, depending on the region where the principal production of acid occurs).

It may be noted, however, that the effects of nitrobenzene are not diminished by raising the pH of the solution to pH 9.5. The same changes in P.D. and resistance occur as at pH 8.1.

In conclusion it may be said that these results, together with those obtained with guaiacol⁶ and hexylresorcinol,¹⁵ make it clear that the behavior of inorganic ions can be greatly altered by organic substances. This important subject deserves further study.

SUMMARY

The effects of nitrobenzene and of benzene resemble those of guaiacol and of hexylresorcinol. The P.D. changes in a positive direction and then in a negative direction. The latter change may bring the P.D. back to the starting point with guaiacol and hexylresorcinol but with nitrobenzene and benzene this is not always the case.

The positive potential change produced by nitrobenzene and benzene may be antagonized to some extent by ammonia.

Nitrobenzene and benzene raise the electrical resistance and this is antagonized to some extent by ammonia.

The results afford a further illustration of the important fact that the behavior of inorganic ions can be changed by organic substances. The apparent mobility of Na^+ is increased and that of K^+ decreased by nitrobenzene and benzene (as is also the case with guaiacol and hexylresorcinol).

¹⁴ Blinks, L. R., *J. Gen. Physiol.*, 1935-36, **19**, 655 ff. At low current densities the resistance is lessened or abolished by addition of ammonia and increased by addition of weak acids. Regarding *Halicystis* see Blinks, L. R., *J. Gen. Physiol.*, 1935-36, **19**, 867.

¹⁵ Osterhout, W. J. V., *J. Gen. Physiol.*, 1939-40, **23**, 569.

THE PRODUCTION OF BACTERICIDAL SUBSTANCES BY AEROBIC SPORULATING BACILLI

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From cultures of an aerobic sporulating bacillus isolated from soil (strain B.G.), there has been crystallized a substance—gramicidin—which exerts a selective bacteriostatic and bactericidal effect against Gram-positive microorganisms (2-5, 9-12). The morphological, cultural, and biochemical characteristics of the organism which produces gramicidin, coincide in general with those of the species described as *Bacillus brevis* in Bergey's Manual of descriptive bacteriology;¹ it must be pointed out, however, that different strains of *B. brevis* vary greatly in the amount of gramicidin which they produce when grown in peptone media, and that strain B.G. from which the substance was first isolated, appears to be one of the most efficient in this respect.

It has been known for a long time that certain microbial cultures exhibit marked antagonistic activities toward other, unrelated species; in particular the antagonistic properties of aerobic, sporulating bacilli have been recognized by a number of workers. Pringsheim (15) described the inhibitory effect exerted by a sporulating bacillus (*Bacillus vulgaris*) on the growth of diphtheria bacilli and other microorganisms on agar media. In 1924 Much (14) observed that a certain strain of *Bacillus mycoides* produced in broth a substance which caused the lysis of several Gram-positive cocci and Gram-negative bacilli. Similar observations were reported by Rosenthal (16-18) who worked with a number of strains of aerobic sporulating bacilli isolated by Duclaux (6) from Cantal cheese, and by Weiland working with a strain of *Bacillus mesentericus* (21).

Recently Stokes and Woodward (19) have described a method for the isolation from soil of microorganisms endowed with bactericidal properties, and reported the preparation from cultures of several of these species of an alcohol-soluble, water-insoluble fraction which carries the bactericidal activity. Finally, from cultures of an aerobic sporulating bacillus Hoogerheide (7, 8, 13) has crystallized a substance which appears identical with gramicidin both in chemical composition and biological activity.

¹ The authors wish to record their indebtedness to Dr. N. Smith, Dr. R. Gordon, and Dr. F. O. Clark of the United States Department of Agriculture, who have generously carried out a number of tests involved in the identification of the culture. More recently Dr. Smith has informed us that the cultures T.C. and LBa mentioned in the present paper are also strains of *B. brevis* whereas the *Tyrophthrix* cultures are strains of *B. subtilis*.

The observations of Much (14) and Rosenthal (16, 18) appear of special interest since these workers reported that their strains exhibited lytic activity not only against Gram-positive organisms, but also against Gram-negative species. An effort was therefore made to isolate from natural sources strains of aerobic sporulating bacilli possessing the biological activities of the cultures studied by Much and Rosenthal.

Isolation of Aerobic Sporulating Bacilli Exhibiting Bactericidal Activity

The material (soil, sewage, manure, cheese, etc.) to be investigated for the presence of aerobic, spore-forming antagonists was heated at 70°C. for 30 minutes to destroy the non-sporulating forms. The heated material was then inoculated into suspensions of living cells of *Escherichia coli* or *Staphylococcus aureus*; these bacterial suspensions, containing approximately 5×10^8 cells per cc., were prepared by resuspending the bacterial cells centrifuged from 8 hour old broth cultures into phosphate buffer (M/15) at pH 7.3. Frequent microscopic and cultural tests were made in an attempt to determine the presence of an antagonistic flora capable of destroying the staphylococci or colon bacilli. Cultures exhibiting antagonistic activity were immediately inoculated into new suspensions of living cells of the same test organisms. In all cases it was found that the addition of small amounts of peptone or gelatin (0.01 per cent) to the bacterial suspension greatly accelerated the disappearance of the staphylococci or colon bacilli. Under optimum conditions, complete disappearance of the staphylococci could often be observed in 18 to 24 hours at 37°C.; it usually took 48 to 72 hours to cause the destruction of the Gram-negative bacilli. At this stage isolation of the active strain of antagonist was readily obtained by heating the mixed culture at 75°C., and plating it on peptone agar.

Many different strains of aerobic sporulating bacilli endowed with properties antagonistic to other microorganisms were isolated by the use of this technique; 7 from soil, 3 from manure, 2 from sewage, and 2 from cheese; all were found active against Gram-positive microorganisms and also, but to a smaller degree, against Gram-negative bacilli. It was also possible to obtain from the Culture Collection of the Lister Institute, London, through the courtesy of its curator, Dr. St. John Brooks, 6 of the *Tyrothrix* cultures isolated by Duclaux in 1887, and tested by Rosenthal. As described by the latter worker, the *Tyrothrix* cultures—especially *Tyrothrix scaber*—were found to exhibit bactericidal properties, although they appear much less active than the cultures isolated in the present work. Finally, it was found that *Bacillus brevis* (strain B.G.) from which gramicidin was first isolated, can also cause the destruction of Gram-negative bacilli resuspended in very dilute (0.003 per cent) peptone solutions.

A complete descriptive study of these different strains of aerobic sporulating bacilli has not been carried out; it can be stated, however, that they appear to belong to different bacterial species since they differ in many

morphological, cultural, and physiological characteristics such as staining reactions, morphology, colony appearance, manner of growth in broth, inhibitory effect of glucose, thermophilic properties, liquefaction of starch, production of bactericidal substances, etc.

Separation of a Soluble Bactericidal Fraction by Extraction of the Cultures with Ethyl Alcohol

It has been shown elsewhere (5) that the bactericidal principles produced by *Bacillus brevis* can be obtained in solution by extracting the cells or peptone cultures of this organism with ethyl alcohol or acetone at acid reaction. The following experiments describe the procedures used to prepare alcoholic solutions possessing bactericidal activity, from cultures of several of the organisms mentioned in the preceding chapter.

The cultures were grown in two different media, (a) 1 per cent tryptone, 0.5 per cent NaCl, tap water—pH 7.0, and (b) 1 per cent gelatin, 0.05 per cent $MgSO_4$, 0.2 per cent KH_2PO_4 , 0.4 per cent Na_2HPO_4 , 0.5 per cent NaCl, tap water—pH 7.0.² The media were distributed in shallow layers (2 cm. thick) and autoclaved at 15 pounds pressure for 30 minutes. They were inoculated with peptone cultures of the selected organism previously heated at 75°C.; 0.5 cc. inoculum was used per liter of medium. Incubation was allowed to proceed for 6 days at 37°C.

At the end of the incubation period, the cultures were adjusted to pH 4.7 with concentrated HCl; this required 3.5 to 4.5 cc. of acid per liter of culture. The acidified cultures were allowed to stand for 24 hours at room temperature; they were then centrifuged and the supernatant fluid discarded. The precipitates were taken up in 95 per cent alcohol, using 50 cc. of this solvent per liter of original culture. On the following day the alcoholic solutions were clarified by filtration through filter paper; they were then diluted with 10 volumes of 1 per cent solution NaCl in tap water. A precipitate formed which contained the active principle; it was separated by filtration and desiccated over P_2O_5 *in vacuo*. The yield of precipitate varied markedly from one culture to another; the largest yields were recovered from cultures of *Bacillus brevis* (strain B.G.), of culture T.C.³ (isolated from a Turkish cheese), and of culture LBa³ (obtained from sewage). Up to 500 mg. of dry material was recovered from 1 liter of culture of these organisms.

The dried material was dissolved in 95 per cent alcohol to give solutions containing 20 mg. per cc. The alcoholic solutions, diluted in distilled water give opalescent colloidal solutions which precipitate on addition of electro-

² When purified gelatin was used, growth was much stimulated by the addition to the medium of small amounts of yeast extract or meat infusion which probably supplied some accessory growth factors.

³ Cultures T.C. and LBa were isolated at the laboratory of the Hospital for Incipient Tuberculosis, Ray Brook, New York, in cooperation with Dr. D. Yagin and Mr. L. Baisden.

lytes. The aqueous solutions exhibit marked bactericidal effect when added to suspensions in buffer solutions of a great variety of microorganisms. They are also effective in protecting mice against infection with pneumococci and streptococci.

Before describing in greater detail the procedure of the bactericidal tests and the results obtained, it appears of interest to report at this time the following observation. As stated above, cultures of the different strains of aerobic sporulating bacilli used in the present study all yield an alcohol-soluble, water-insoluble fraction endowed with bactericidal activity; the yield of this material varied enormously from one culture to the other (from 20 mg. to 500 mg. per liter of medium) but surprisingly enough, the bactericidal activity per unit weight of the different preparations thus obtained appeared of the same order. In all cases, for instance, it took approximately 0.01 mg. of the dry materials to kill 10^{10} staphylococci in 5 hours at $37^{\circ}\text{C}.$; approximately 0.01 mg. of material, administered intraperitoneally, was sufficient to protect mice against 10,000 fatal doses of Type I pneumococcus. The similarity in solubility properties, and in biological activity, of the material obtained from the different cultures, suggests that the strains of sporulating bacilli used in the present study all produce in different amounts similar types of substances endowed with bactericidal activity. In fact, it can be stated at the present time that a substance apparently identical with gramicidin in crystalline structure, analytical composition, and biological properties, has been isolated from culture T.C. which, in many growth characteristics, differs markedly from *Bacillus brevis* (strain B.G.) from which gramicidin was first isolated. Culture T.C. also yields another bactericidal substance similar to, if not identical with tyrocidine, also crystallized from cultures of *Bacillus brevis* (strain B.G.).

Gramicidin and tyrocidine are two crystalline substances which have been separated by differential solubilities in acetone-ether mixtures from the alcohol-soluble, water-insoluble fraction obtained from cultures of *Bacillus brevis* (strain B.G.) (9). Although both substances are essentially polypeptides consisting in part of *d*-amino acids, they exhibit differences in chemical composition which have been considered elsewhere (9); they also differ markedly in biological properties, and some of these biological differences will be described in the following experiments.

Bactericidal Activity in Vitro of Gramicidin and Tyrocidine

Five hours old cultures of *Escherichia coli* and *Staphylococcus aureus* in meat infusion peptone broth were centrifuged and the cells resuspended in two different media; (a)

m/15 mixed phosphate buffer, pH 7.3; (b) supernatant of the broth culture from which the *E. coli* cells had been collected; this supernatant fluid was filtered through a Berkefeld candle and adjusted to pH 7.3 before use. The bacterial suspensions (in buffer or metabolized broth) gave approximately 3×10^9 colonies per cc. when plated in meat infusion peptone agar. Graded amounts of gramicidin and tyrocidine, diluted in distilled water, were added to 3 cc. volumes of the bacterial suspensions. The mixtures were incubated at 37°C. and streaked on meat infusion peptone agar after 3 hours and

TABLE I
The Effect of Gramicidin and Tyrocidine on Bacterial Suspensions in Vitro

| Amount of substance added to 3 cc. bacterial suspension | | Bacterial suspension in buffer | | | | Bacterial suspension in metabolized broth | | | |
|------------------------------------------------------------|-------|--------------------------------|--------------------|----------------|--------------------|----------------------------------------------|--------------------|----------------|--------------------|
| | | Growth on agar plates* | | Lysis† | | Growth on agar plates | | Lysis | |
| | | <i>E. coli</i> | Staphy- lococci | <i>E. coli</i> | Staphy- lococci | <i>E. coli</i> | Staphy- lococci | <i>E. coli</i> | Staphy- lococci |
| Gramicidin | mg. | | | | | | | | |
| | 0.500 | ++++ | — | 0 | 0 | ++++ | — | 0 | 0 |
| | 0.100 | ++++ | — | 0 | 0 | ++++ | — | 0 | 0 |
| | 0.010 | ++++ | — | 0 | 0 | ++++ | — | 0 | 0 |
| | 0.005 | ++++ | — | 0 | 0 | ++++ | + | 0 | 0 |
| | 0.002 | ++++ | + | 0 | 0 | ++++ | +++ | 0 | 0 |
| Tyrocidine | 0.500 | — | — | L | L | ++++ | — | 0 | L |
| | 0.100 | — | — | L | L | ++++ | — | 0 | L |
| | 0.050 | — | — | L | L | ++++ | — | 0 | L |
| | 0.025 | +++ | — | L | L | ++++ | ++++ | 0 | L |
| | 0.010 | ++++ | ++++ | 0 | 0 | ++++ | ++++ | 0 | 0 |
| Controls | 0 | ++++ | ++++ | 0 | 0 | ++++ | ++++ | 0 | 0 |

* +++++ = abundant growth on meat infusion peptone agar.

— = no " " " " " "

† L = destruction of cellular structure as revealed by microscopic analysis; large amounts of tyrocidine cause a precipitation of cellular material which masks the lytic phenomenon.

0 = no lysis.

12 hours incubation. The lytic effect of gramicidin and tyrocidine on the bacterial cells was also determined by microscopic examination. Although the results of growth on agar plates were about the same when the mixtures were cultured after 3 hours or 12 hours incubation, lysis was not evident at the first period of observation. The results presented in Table I report growth on agar plates and lysis of the bacterial cells after the mixtures of bacterial suspensions and gramicidin or tyrocidine had been incubated for 12 hours.

The results presented in Table I confirm the great activity of gramicidin against staphylococcus, a Gram-positive organism, and its ineffectiveness against *E. coli*, a Gram-negative species. Tyrocidine, on the contrary,

exhibits bactericidal activity against both test organisms, resuspended in buffer solutions. This correlation between the reaction of the cell to the Gram stain and its differential susceptibility to gramicidin has been extended to a number of other bacterial species; pneumococci, streptococci, staphylococci, diphtheria and diphtheroid bacilli, aerobic and anaerobic sporulating Gram-positive bacilli, have all been found to be susceptible to both gramicidin and tyrocidine. On the contrary, the following Gram-negative groups, *Escherichia*, *Klebsiella*, *Shigella*, *Salmonella*, *Hemophilus*, *Neisseria*, are resistant to gramicidin but susceptible to tyrocidine.

Another generalization appears justified at the present time. Treatment with tyrocidine often results in the lysis of the bacterial cells (streptococci, diphtheria and diphtheroid bacilli are an exception to this rule). On the contrary, when the cells of susceptible bacterial species are treated with gramicidin, they retain their characteristic morphology and staining reactions long after they have lost the capacity to grow when inoculated into plain broth or on plain agar.

It is also apparent from the results presented in Table I that both gramicidin and tyrocidine are more effective when tested in buffer solutions than in the presence of the constituents of meat infusion peptone broth. In particular, the activity of tyrocidine against *E. coli* (and other Gram-negative bacilli) is remarkably inhibited when the bacterial cells are resuspended in peptone solutions or meat infusion peptone broth.

The Activity of Gramicidin and Tyrocidine against Bacterial Infections

As reported elsewhere (9, 10), the intraperitoneal injection of gramicidin exerts a protective action against infection of mice with pneumococci and streptococci; gramicidin is ineffective *in vitro* against Gram-negative bacilli and also fails to protect mice against infection with *Klebsiella pneumoniae*. Tyrocidine, on the contrary, can be shown to exert a bactericidal effect *in vitro* against Gram-negative as well as Gram-positive microorganisms; however, all attempts to obtain a protective effect with this substance against Gram-negative infections have so far failed.

Varying amounts of tyrocidine have been administered to mice by the intraperitoneal, subcutaneous, intravenous, or oral routes, and failed to protect these animals against infection with *Klebsiella pneumoniae* or *Salmonella aertrycke*. In fact, the feeding of large amounts of tyrocidine to mice even failed to modify the normal Gram-negative intestinal flora. It can be stated in passing that large amounts of young active cultures of aerobic sporulating bacilli (*Bacillus brevis* strain B.G., culture T.C., and *Tyrothrix scaber*) have been fed to mice and guinea pigs in an attempt to

modify the intestinal flora as suggested by Rosenthal (17); it was indeed possible to recover these bacterial species from the feces for a number of days or even weeks after these cultures had been fed to guinea pigs, showing that the sporulating bacilli had become established in the intestinal tract. There was also definite indication that the Gram-positive components of the normal intestinal flora had been displaced by the aerobic sporulating bacilli, but in no case could we observe any significant reduction of the number of coliform bacilli.

TABLE II
The Protective Effect of Gramicidin and Tyrocidine against Infection of Mice with Type I Pneumococcus*

| Treatment (Intraperitoneal) | | Infecting dose of pneumococci† | | | | | | | | | | | |
|-----------------------------|-------|--------------------------------|----|----|----|----|----|------------------|----|----|------------------|----|----|
| | | 10 ⁻⁴ | | | | | | 10 ⁻⁷ | | | 10 ⁻⁸ | | |
| | mg. | | | | | | | | | | | | |
| Gramicidin | 0.025 | S | S | S | S | S | S | — | — | — | — | — | — |
| | 0.01 | S | S | S | S | S | S | — | — | — | — | — | — |
| | 0.005 | S | S | S | S | S | S | — | — | — | — | — | — |
| | 0.002 | S | S | S | S | S | S | — | — | — | — | — | — |
| Tyrocidine | 0.250 | D4 | D5 | S | S | S | S | — | — | — | — | — | — |
| | 0.100 | D5 | S | S | S | S | S | — | — | — | — | — | — |
| | 0.050 | D4 | D4 | D5 | S | S | S | — | — | — | — | — | — |
| | 0.025 | D2 | D2 | D4 | D4 | D5 | D8 | — | — | — | — | — | — |
| Controls | 0 | | | | | | | D2 | D4 | D5 | D4 | D4 | D5 |

* In this particular experiment all mice treated with gramicidin were alive and well when discarded 9 days after inoculation. Usually a few scattered deaths are observed whatever the dose of gramicidin used for treatment.

† S = survival of the animal.

D = death " " " Numeral indicates number of days elapsing between inoculation and death.

All these observations would indicate that, like other classical antiseptics, tyrocidine is essentially ineffective *in vivo*. Surprisingly enough, however, crystalline preparations of this substance can exert a definite protective action against pneumococcus infections in mice. This is illustrated in the following experiment.

Mice were infected intraperitoneally with 10,000 fatal doses of *Pneumococcus* Type I; within 15 minutes after infection they were treated intraperitoneally with varying amounts of gramicidin or tyrocidine diluted in distilled water.

The results presented in Table II show that one single injection of 0.050 to 0.100 mg. of tyrocidine administered intraperitoneally is sufficient to

protect mice against 10,000 fatal doses of pneumococcus; tyrocidine is however much less active than gramicidin, since the same protective effect could be obtained with 0.002 mg. of the latter substance.

Gramicidin and tyrocidine differ in many other biological properties; for instance 0.3 to 0.5 mg. of gramicidin injected intraperitoneally is sufficient to kill a 25 gm. mouse in 48 hours; 2 mg. of tyrocidine is required for the same toxic effect; the latter substance therefore is less toxic than the former but it will be recalled that it is also much less effective against the Gram-positive bacterial cell both *in vitro* and *in vivo*.

TABLE III
Hemolytic Activity of Gramicidin and Tyrocidine in Vitro

| Bactericidal agent | | 1 cc. of 10 per cent washed red cells—Hemolysis after incubation for the following lengths of time: | | | |
|--------------------|-------|-----------------------------------------------------------------------------------------------------|--------|--------|---------|
| | | 15 min. | 3 hrs. | 8 hrs. | 24 hrs. |
| Gramicidin | mg. | | | | |
| | 0.400 | — | — | — | — |
| | 0.200 | — | — | — | — |
| | 0.100 | — | — | — | — |
| | 0.050 | — | — | — | — |
| | 0.020 | — | — | — | — |
| Tyrocidine | 0.400 | ++++ | ++++ | ++++ | ++++ |
| | 0.200 | ++++ | ++++ | ++++ | ++++ |
| | 0.100 | +++ | +++ | +++ | +++ |
| | 0.050 | + | ++ | ++ | ++ |
| | 0.020 | — | + | + | + |
| Control..... | 0 | — | — | — | — |

++++ = complete hemolysis.

— = no hemolysis.

Studies of the effect of gramicidin and tyrocidine on the physiological functions of the susceptible bacterial cells have also revealed profound differences in the mechanisms of action of the two substances; these studies will be reported later. At this time, mention will be made only of the effect of the two bactericidal substances on the mammalian erythrocyte.

Hemolytic Action of Gramicidin and Tyrocidine in Vitro.—Rabbit erythrocytes were washed free of serum and resuspended in a volume of 5 per cent aqueous solution of glucose sufficient to give a concentration of cells corresponding to 1/10 that of the blood. Graded dilutions of gramicidin and tyrocidine in 10 per cent glucose were added to the cell suspension and the mixtures incubated at 37°C. Hemolysis readings were made after 15 minutes, 3 hours, 8 hours, and 24 hours incubation.

As shown in Table III tyrocidine causes an immediate hemolytic effect which does not increase appreciably with prolonged incubation. On the contrary, no hemolytic effect could be observed with gramicidin, even after 24 hours incubation.

DISCUSSION

The antagonism exerted by certain types of microorganisms against other microbial species is a fact of common observation (12, 20) but the mechanism of the antagonistic action may vary so profoundly from one case to another that it hardly permits of any general systematic formulation. "Antibiosis" (12) may be due, for instance, to competition for oxygen or other*essential nutrients, to liberation into the culture medium of acidic or basic products which interfere with growth, to the production of other metabolites which may kill the cells, etc., etc. The antagonistic action of certain aerobic sporulating organisms discussed in the present paper, offers on the contrary a fairly well defined entity. From a great variety of sources (soil, sewage, manure, cheese, etc.) strains can be isolated of aerobic sporulating bacilli, differing in morphological, cultural, and physiological characteristics, which all produce in peptone media an alcohol-soluble, water-insoluble fraction endowed with bactericidal activity. Among the first saprophytic, aerobic sporulating bacilli to be described, were those isolated by Duclaux (6) from Cantal cheese; on account of their origin, Duclaux gave to these organisms the generic name of *Tyrothrix* (now to be placed in the genus *Bacillus*). In 1925 Rosenthal (16, 18) showed that the strains of *Tyrothrix* isolated by Duclaux slowly release into the culture medium a substance endowed with lytic and bacteriostatic activity. The antagonistic action recognized by Rosenthal was probably due to the alcohol-soluble, water-insoluble fraction described in the present and other reports. The name tyrothricin has been proposed for this alcohol-soluble, water-insoluble fraction (10).

Tyrothricin has now been obtained by growing different species of aerobic sporulating bacilli on several media; (a) tryptone solution, a medium rich in tyrosine and tryptophane, (b) gelatin solution, a medium deficient in these aromatic amino acids, (c) synthetic media, consisting of mixtures of amino acids, with or without tryptophane and tyrosine. The yields of tyrothricin have varied considerably on the different media with the different organisms. It seems worth reporting that very large yields have been obtained by growing *Bacillus brevis* (strain B.G.) in a gelatin medium. Since gelatin is deficient in aromatic amino acids, and since tyrothricin is rich in tyrosine and tryptophane, it is evident that the organism is capable of rapidly syn-

thesizing large amounts of these aromatic amino acids. It will be recalled also that many of the amino acids which constitute tyrothricin are of the unnatural *d*-type; since the *d*-amino acids are not present in gelatin, it appears that these substances are also synthesized by the bacillus in the course of its growth.

Crude tyrothricin is bactericidal *in vitro* not only against Gram-positive microorganisms, but also against Gram-negative species. Failure to recognize this fact in earlier publications was due to the following reasons: (a) the activity of the crude product is very much greater against Gram-positive than against Gram-negative species; (b) the activity against Gram-negative bacilli is markedly inhibited in the presence of broth constituents, and all the earlier bactericidal tests were carried out directly in broth cultures.

Tyrothricin, prepared from *Bacillus brevis* (strain B.G.) has yielded two crystalline products, the chemical nature of which has been outlined elsewhere (9, 10). One of these substances has been called gramicidin on account of its selective bacteriostatic and bactericidal effect against Gram-positive microorganisms. The other substance is an organic base which has been called tyrocidine to recall the generic name of *Tyrothrix* and because the substance is rich in the amino acid tyrosine.

In spite of their common origin and of the fact that both substances are polypeptides, gramicidin and tyrocidine differ not only in certain chemical properties, but also in biological activity. Gramicidin is effective only against Gram-positive microorganisms; tyrocidine, when tested in buffer solution in the absence of broth, affects both Gram-positive and Gram-negative species. Tyrocidine causes immediate hemolysis of washed red cells, whereas gramicidin has no hemolytic effect. Tyrocidine also causes lysis of many bacterial species; there is definite evidence, however, that the lytic effect in this case is not a direct one, but is only a secondary autolytic process which follows upon death of the cell (2).

Although the effect of gramicidin is to some extent inhibited by the presence of peptones and serum, this inhibitory effect is especially marked in the case of tyrocidine; in fact, it is very difficult to recognize any effect of tyrocidine on Gram-negative bacilli when these organisms are suspended in peptone solutions.

It will be shown elsewhere that tyrocidine immediately destroys the metabolic activity not only of bacterial but also of animal cells. This effect can be recognized by the immediate loss of oxygen uptake, of acid production, of reducing ability. On the contrary these essential metabolic functions are respected by gramicidin even in the case of the most susceptible bacterial cells.

All available evidence, therefore, indicates that tyrocidine behaves like a general protoplasmic poison, whereas the effect of gramicidin is of a much more subtle nature. In fact it will be shown elsewhere that the effect of gramicidin is to some extent reversible. For instance staphylococci "killed" with gramicidin and which are unable to grow on meat infusion peptone media can be made to grow in the presence of certain tissue components (1).

Since gramicidin is not a gross protoplasmic poison, and since it is less inhibited by peptones than are most antiseptics, it becomes easier to understand why under certain conditions it retains much of its activity in the presence of animal tissues. In fact, gramicidin, when applied locally at the site of the infected area, does exhibit a definite activity against infection with pneumococci and streptococci (9, 11, 12). It appears, however, that gramicidin is almost completely inactive against systemic infection when injected intravenously (4). Whether this ineffectiveness is due to physical properties which prevent diffusibility of the substance throughout the tissues or whether it is due to the inhibitory effect of tissue components upon its activity, cannot be decided at the present time.

Tyrocidine, although inactive against infection with Gram-negative bacilli, appears to exhibit definite activity against pneumococcus infection in mice. The results reported in Table II have been obtained with preparations recrystallized several times and can hardly be explained by a contamination of tyrocidine with gramicidin. Tyrocidine is much less active than gramicidin against pneumococcus infections in mice; on the other hand, when tested *in vitro* against these same microorganisms resuspended in buffer solutions, tyrocidine is almost as active as gramicidin. This discrepancy appears of special interest since it offers a concrete example of two substances having a common origin, definite similarity in chemical structure, but differing widely in "chemotherapeutic" action. It is hoped that a comparison of the chemical structure of the two substances, and a knowledge of the mechanism of their physiological action against bacterial and tissue cells, may throw light on some of the factors which govern the effectiveness of antiseptic agents in the animal body.

SUMMARY

Several species of aerobic sporulating bacilli recently isolated from soil, sewage, manure, and cheese, as well as authentic strains obtained from type culture collections, have been found to exhibit antagonistic activity against unrelated microorganisms.

Cultures of these aerobic sporulating bacilli yield an alcohol-soluble, water-insoluble fraction,—tyrothricin,—which is bactericidal for most Gram-positive and Gram-negative microbial species.

Two different crystalline products have been separated from tyrothricin. One, which may be called tyrocidine, is bactericidal *in vitro* for both Gram-positive and Gram-negative species; the other substance, gramicidin, is effective only against Gram-positive microorganisms. In general, tyrocidine behaves like a protoplasmic poison and like other antiseptics, loses much of its activity in the presence of animal tissues. Gramicidin on the contrary exerts a much more subtle physiological effect on the susceptible bacterial cells and, when applied locally at the site of the infection, retains *in vivo* a striking activity against Gram-positive microorganisms.

Addendum.—Heilman and Herrell (*Proc. Soc. Exp. Biol. and Med.*, 1941, **46**, 182) have recently described a marked hemolytic effect of gramicidin in tissue culture, whereas, in the experiments reported in the present paper, no hemolysis was observed when washed sheep red cells were resuspended in isotonic glucose solution. We have now established that gramicidin does indeed cause a slow hemolysis of erythrocytes resuspended in buffer or saline solutions, but the addition of small amounts of glucose to the system is sufficient to prevent any hemolytic action.

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CHEMOIMMUNOLOGICAL STUDIES ON THE SOLUBLE SPECIFIC SUBSTANCE OF PNEUMOCOCCUS

V. THE STRUCTURE OF THE TYPE III POLYSACCHARIDE

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It is the capsular polysaccharide which endows the pneumococcus with type specificity and it is this same substance which envelops the bacterial cell and renders it difficult of destruction by the phagocytes of the invaded host. When rabbits are injected with suspensions of these heat-killed, intact microorganisms, the various antibodies engendered are directed toward a number of the constituents of the bacterium. But it is the antibody elicited by the capsular polysaccharide antigen which confers type-specific immunity on experimental animals against infection with virulent pneumococci. Presumably the antibody combines chemically with the capsular polysaccharide *in situ* and renders the microorganisms susceptible to destruction by the natural protective mechanisms of the host (1).

This remarkable property of specificity exhibited by the many pneumococcal types is conditioned solely by the chemical constitution of the individual carbohydrate molecules which constitute the encapsulating material. It is, therefore, only through a knowledge of their intricate chemical make-up that one can eventually gain an understanding of the factors which govern the specificity of the bacterial polysaccharides and of the immunobiological properties of the microorganisms from which they are derived.

The capsular polysaccharide of Type III pneumococcus can be obtained from bacterial cultures in a high state of purity (2). The substance is a polybasic acid, non-diffusible, and is constituted from the elements carbon, hydrogen, and oxygen. Considerable progress in the elucidation of the structure of the polysaccharide

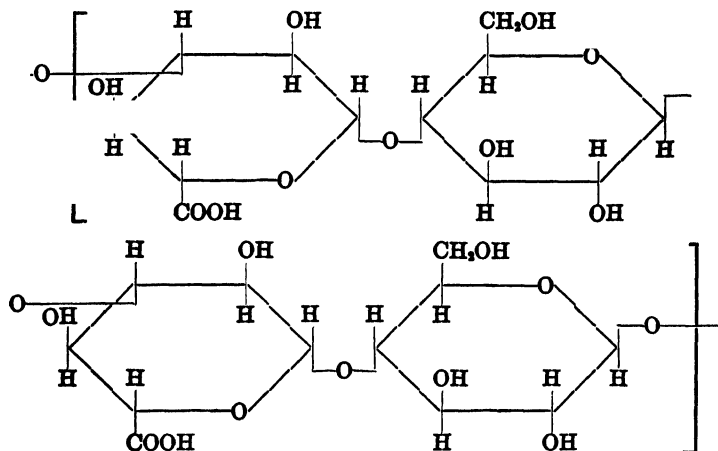
was made when it was shown to be built up from units of an aldobionic acid, 4- β -glucuronosidoglucose or cellobiuronic acid (3). In the intact carbohydrate the cellobiuronic acid units are linked through the reducing group of the aldobionic acid to one of the hydroxyl groups of a similar unit. The objective of the present investigation is to determine the position of the intermolecular linkage of the cellobiuronic acid units.

The procedure of experimentation is that which is well established and so successfully used in studying the structure of carbohydrates in general; namely, methylation of the free hydroxyl groups of the polysaccharide followed by acid hydrolysis and identification of the partially methylated sugars found in the hydrolysate. In the present investigation we have found it advisable first to reduce catalytically the fully methylated polysaccharide. The carbomethoxyl groups of the methylated polysaccharide are thus converted to primary carbinol groups. On hydrolysis of this methylated reduced polysaccharide the uronic acid constituent appears in the hydrolysate as a dimethylglucose, whereas the glucose constituent of the aldobionic acid unit of the parent polysaccharide is isolated as 2,3,6-trimethylglucose.

The specific polysaccharide of Type III pneumococcus is readily methylated with dimethyl sulfate and sodium hydroxide and the carboxyl groups esterified with diazomethane. In this way a neutral and completely methylated polysaccharide is obtained. This substance is now reduced at high temperature and pressure, with barium-copper chromite as catalyst. Hydrolysis of this product yields equimolecular amounts of di- and trimethylglucose. These substances are converted to the corresponding methylglucosides and separated by the usual procedures. Hydrolysis of the trimethylmethylglucosides yields the known crystalline 2,3,6-trimethylglucose. From the dimethylmethylglucoside portion two crystalline products have been obtained. One of these is identical with an authentic sample of a synthetic 2,4-dimethyl- β -methylglucoside (4); the second crystalline product has been identified as the isomeric α form.

With the identification of 2,4-dimethyl- α - and β -methylglucosides as products of hydrolysis of the reduced methylated polysaccharide it becomes apparent that the methylated aldobionic acid units are linked through position 3 of the methylated glu-

curonic acid. Consideration of the data now at hand supports a structure of the Type III pneumococcus polysaccharide wherein glucose is linked to the 3rd carbon atom of the glucuronic acid while the latter in turn is known to be linked to the 4th carbon atom of a second glucose molecule (3).



In the accompanying graphic formula the glucuronosidic linkage has been definitely established as having the β configuration. The configuration of the linkage between the aldobionic acid units (*i.e.*, the glucosidic linkage) is assumed to be of the same type. The justification for this resides in the fact that the specific polysaccharide itself is levorotatory and on acid hydrolysis, under conditions in which the glucosidic, but not the glucuronosidic linkages, are attacked, the rotation of the solution changes from levo to dextro (2).

It is not unusual to find naturally occurring polysaccharides in which the glycosidic linkage is in position 3 (5). Most notable of those in which glucose is linked in position 3 is the polysaccharide isolated by Zechmeister and Toth from yeast cells (6). In the case of the Type III pneumococcus carbohydrate it is of interest to find the linkages of the saccharide units alternating between positions 3 and 4.

With the elucidation of this problem we have established the chemical constitution of the first of the capsular polysaccharides of an important group of pathogens. It is hoped that further work

in this field will eventually give us a more thorough understanding of the chemical basis underlying the specificity of the many types of pneumococci.

EXPERIMENTAL

Methylation of Type III Polysaccharide—10.0 gm. of the polysaccharide were dissolved in 100 cc. of 0.3 N sodium hydroxide and methylated with dimethyl sulfate and sodium hydroxide in the usual manner (7). The solution was neutralized with dilute sulfuric acid, cooled to 50°, acidified with 4 cc. of 10 N sulfuric acid, and the heavy precipitate extracted with warm butanol. The butanol extract was washed free from sulfate ions and the methylated polysaccharide recovered by evaporating the solvent *in vacuo*. The residual methylated carbohydrate was dissolved in acetone and precipitated when poured into cold petroleum ether. 12.1 gm. of material were recovered. The product had a methoxyl content of 33.85 per cent and contained some sodium ions. 10.9 gm. of this material were remethylated, yielding 9.7 gm. of a product having a methoxyl content of 36.55 per cent. This material was further methylated with methyl iodide and silver oxide (8). 8.2 gm. of the methyl ester of the methylated polysaccharide were recovered. Because this material was still low in methoxyl content, the product was acetylated with pyridine and acetic anhydride. The product obtained from this reaction was dissolved in acetone and treated with dimethyl sulfate and sodium hydroxide in the usual manner. 7.6 gm. of the methylated polysaccharide were finally obtained.

Analysis— $[C_{11}H_{12}O_4(OCH_3)_5 COOH]_n$

Calculated. CH_2O 38.0, acid equivalent 408

Found. " 37.5, " " 413

Rotation— $[\alpha]_D^{25} = -35.8^\circ$ ($c = 2.0\%$ in $CHCl_3$ -absolute alcohol 4:1)

Methyl Ester of Methylated Polysaccharide—7.6 gm. of the above product were dissolved in 400 cc. of methanol. An ethereal solution of diazomethane was added in slight excess. The solvents were removed and 7.96 gm. of an amorphous product melting at 185–200° were recovered. The methyl ester of the methylated polysaccharide is soluble in the usual organic solvents with the

exception of petroleum ether. The material is only slightly soluble in water.

Analysis— $[C_{13}H_{30}O_{11}]_n$.

Calculated, CH_2O 44.08; found, CH_2O 43.35

Saponification with Dilute Alkali—Calculated. Equivalent weight 422
Found. " " 423

Rotation— $[\alpha]_D^{25} = -36.8^\circ$ in $CHCl_3$ ($c = 1.0\%$)

Catalytic Reduction of Methylated Polysaccharide—1.06 gm. of the methylated polysaccharide methyl ester were dissolved in 75 cc. of methanol and 1 gm. of barium-copper chromite catalyst (9) was added. Reduction of the polysaccharide was carried out with hydrogen at 175° under a pressure of 3200 pounds per sq. inch. Complete reduction was effected in 20 hours. After completion of the reaction the catalyst was removed by filtration and the solution treated with norit, filtered, and evaporated to dryness. The residue weighing 800 mg. was a brittle, colorless solid insoluble in ether and petroleum ether, but soluble in chloroform, alcohol, and cold water. The substance reduced Fehling's solution only after acid hydrolysis, and the hydrolysate failed to give the naphthoresorcinol test for uronic acid. A sample of the reduced material failed to react with dilute alkali, indicating the absence of ester groups.

$[C_{17}H_{30}O_{10}]_n$. Calculated, CH_2O 39.3; found, CH_2O 37.9

Rotation— $[\alpha]_D^{25} = -31.0^\circ$ in H_2O ($c = 0.7\%$)

$[\alpha]_D^{25} = -15.6^\circ$ in $CHCl_3$ ($c = 0.6\%$)

Hydrolysis of Reduced Methylated Polysaccharide—290 mg. of the reduced polysaccharide were dissolved in 2 cc. of cold concentrated hydrochloric acid and allowed to stand at room temperature overnight. The solution was then diluted to 10 cc. with water and boiled under a reflux for 6 hours. After decolorization with norit and filtration, the colorless solution was neutralized with barium carbonate and concentrated to dryness *in vacuo*. Extraction of the dry salts with acetone yielded 298 mg. of a colorless syrup. The latter, which contained the partially methylated hydrolytic products, was heated in a sealed tube at $70-75^\circ$ with methanol containing 1 per cent dry hydrogen chloride. The solution was neutralized with solid barium carbonate, and

most of the barium chloride which formed was precipitated by the addition of a large amount of acetone. The solution was evaporated to dryness and the residue dissolved in water. The products of reaction were partitioned between chloroform and water, the trimethylmethylglucosides dissolving in the chloroform phase and the dimethylmethylglucosides remaining in the aqueous phase.

After hydrolysis of the product from the chloroform extract a 50 per cent yield of crystalline 2,3,6-trimethylglucose was obtained. The derivative melted at 113° and the melting point was not depressed when the substance was mixed with an authentic sample.

$C_8H_{18}O_6$. Calculated, CH_2O 41.9; found, CH_2O 40.8

Rotation— $[\alpha]_D^{25} = +69.70^{\circ}$, equilibrium in H_2O ($c = 1.3\%$)

The aqueous phase was evaporated to dryness, yielding 117 mg. of crude dimethylmethylglucosides. When this material was dissolved in 2 cc. of ether and allowed to stand in the ice chest, 69 mg. of crystalline material separated and two different crystalline forms were obviously present. Repeated recrystallization from ether gave 23 mg. of pure 2,4-dimethyl- β -methylglucoside, melting at $122-123^{\circ}$. Upon cooling the substance crystallized and remelted at $105-107^{\circ}$. These melting point values were not depressed when the above product was mixed with synthetic 2,4-dimethyl- β -methylglucoside. On two other occasions a similar yield of this substance was obtained upon hydrolysis of the reduced methylated polysaccharide.

$C_8H_{18}O_6$. Calculated, CH_2O 41.9; found, CH_2O 41.2

Rotation— $[\alpha]_D^{25} = -16.5^{\circ}$ in acetone ($c = 0.5\%$)

After separation of as much 2,4-dimethyl- β -methylglucoside as possible the mother liquors on long standing in the ice box deposited 15 mg. of crystals in the form of clusters. This substance melted at $79-81^{\circ}$. When mixed with 2,3-dimethyl- α -methylglucoside (10) (m.p. $83-84^{\circ}$), the mixture melted at $65-75^{\circ}$, but when mixed with an authentic sample of 2,4-dimethyl- α -methylglucoside, the melting point was not depressed.

$C_8H_{18}O_6$. Calculated. C 48.65, H 8.11, CH_2O 41.89

Found. " 48.45, " 7.92, " 41.66

Rotation— $[\alpha]_D^{25} = +159^{\circ}$ in acetone ($c = 0.3\%$)

The sample taken for optical rotation measurement was observed to be contaminated with a small amount of the crystals of the β -methylglucoside.

2,4-Dimethyl- α -Methylglucoside—By methylation of 6-trityl- α -methylglucoside and subsequent detritylation Robertson and Waters obtained, in addition to 2,3,4-trimethyl- α -methylglucoside, a substance of unknown structure which melted at 79° (11). A sample of the latter material was therefore prepared. On purification the material was found to be identical with the high rotating dimethylmethylglucoside obtained by us from the hydrolytic products of the reduced methylated Type III polysaccharide. The substance melted at 79–80° and the melting point was depressed when mixed with known 2,3-dimethyl- α -methylglucoside.

| | | | | |
|------------------|-------------|----------|---------|---------------|
| $C_8H_{18}O_6$. | Calculated. | C 48.65, | H 8.11, | CH_3O 41.89 |
| | Found. | " 48.89, | " 8.16, | " 41.73 |

Rotation— $[\alpha]_D^{25} = +186^\circ$ in acetone ($c = 1.0\%$)

Conversion of 2,4-Dimethyl- α -Methylglucoside to β Form—A solution of 110 mg. of the new dimethyl- α -methylglucoside in 2 cc. of methanol containing 2.5 per cent HCl was heated in a sealed tube at 100° for 16 hours. The solvent was removed *in vacuo* and the residue twice evaporated after the addition of small amounts of toluene. When this product was dissolved in ether, the solution yielded 15.9 mg. of crystals melting at 121.5–123° (corrected). The product was identical with the high melting dimethylmethylglucoside obtained from the hydrolytic products of the methylated Type III polysaccharide and was also identical with the dimethyl- β -methylglucoside recently synthesized by an entirely different procedure (4). All of these substances therefore have their fixed methyl groups in the same position. By the following experiments they are shown to be derived from 2,4-dimethylglucose.

Proof of Structure of 2,4-Dimethyl- α - and β -Methylglucosides—It has been shown by Brigl and Schinle (12) that osazones can be formed from aldoses methylated in position 2. This reaction involves the removal of the methoxyl group and is regarded as evidence that the methoxyl was originally substituted in position 2. In order to establish the structure of the 2,4-dimethylglucose derivatives isolated in this study, the latter were converted to

the known 4-methylglucosazone. In this manner final proof of the structure of these derivatives was established.

The 94.1 mg. of mixed glucosides remaining after isolation of the crystalline β -methylglucoside in the previous experiment were hydrolyzed in a sealed tube at 100° for 16 hours. 320 mg. of crystalline sodium acetate and 184 mg. of phenylhydrazine hydrochloride were now added. The solution, after removal of a small precipitate, was heated on a water bath for 2.5 hours. The oil which separated was dissolved in dilute alcohol. The crystals of osazone which separated on cooling were recrystallized from benzene. This osazone melted at 156–157° (corrected) and the melting point was not depressed when mixed with authentic 4-methylglucosazone.

| | | | |
|--------------------------------------------------------------------------------------|-------------|-------------------|------|
| [C ₁₈ H ₂₁ O ₅ N ₄ (OCH ₃)]. | Calculated. | CH ₃ O | 8.34 |
| | Found. | " | 7.95 |

4-Methylglucosazone—Crystalline 4-methyl-2,3,6-triacetyl- β -methylglucoside (13) was hydrolyzed and treated with sodium acetate and phenylhydrazine hydrochloride under the conditions described above. The osazone crystallized from dilute alcohol and was recrystallized from benzene. It melted at 156–157° (corrected). Munro and Percival (14) and Schinle (15) have observed 158° and 159°, respectively, as the melting point of 4-methylglucosazone.

SUMMARY

1. The methylation of the capsular polysaccharide of Type III pneumococcus and its catalytic reduction have been described.

2. Hydrolysis of the reduced polysaccharide yields the known 2,3,6-trimethylglucose and two substances identified as 2,4-dimethyl- α - and β -methylglucosides. Both of these substances have been synthesized and their structures confirmed by an independent series of reactions.

3. From the hydrolysis products of the reduced methylated polysaccharide it has been possible to establish the position of linkage between the aldobionic acid units in the intact polysaccharide.

The catalytic reductions were carried out with the generous cooperation of the late Dr. P. A. Levene to whom the authors are also

indebted for the sample of 4-methyl-2,3,6-triacetyl- β -methylglucoside.

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THE EFFECT OF A POLYSACCHARIDE-SPLITTING ENZYME ON STREPTOCOCCAL INFECTION

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PLATE 26

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When Kendall, Heidelberger, and Dawson (1) isolated a polysaccharide from group A hemolytic streptococcus cultures, they gave renewed interest to the subject of streptococcus capsules. Somewhat later, Seastone (2) showed that the capsules of group C streptococci were chemically similar to those of group A. In both groups, this capsular polysaccharide is non-type-specific; with heat-killed vaccines prepared from both group A and group C strains, it has been impossible to stimulate in rabbits the production of any antibodies against this capsular substance. A further interesting fact is that a chemically similar substance occurs widely throughout the mammalian organism, and has been isolated by Meyer and his collaborators from vitreous humor, Wharton's jelly, synovial fluid, and the fluid from a mesothelial tumor (3-5). Moreover, enzymes capable of splitting this polysaccharide occur in certain bacteria (6, 7) and also in some mammalian tissues (8, 7).

All of the above facts raise interesting questions concerning the rôle of the capsule in infection with streptococci. How may a capsule, made up of a substance normally present in the host, influence the pathogenesis and virulence of an invading streptococcus? It is with this question that the present paper is principally concerned.

Preparation of the Capsular Polysaccharide

Kendall, Heidelberger, and Dawson obtained the polysaccharide from the supernatant fluids of broth cultures of group A streptococci following centrifugation. Upon repeating their work, we found that all of our preparations contained about 50 per cent of blood group A substance. This contaminating material has been shown to be present in large quantities in peptone (9). In order to eliminate this and other sources of error, all the media, before they were used for culture, were prepared as follows:—

The medium was essentially that of Todd and Hewitt (10) except that beef heart or vegex was substituted for the horse meat, and the alcohol-insoluble fraction was

removed before use. The beef heart infusion for 60 liters of medium was first concentrated *in vacuo* to 4 liters; when vegex was used 600 gm. of the paste was dissolved in 4 liters of water. In this concentrate 1,500 gm. of Pfanstiehl peptone was dissolved, then 2.5 volumes of ethyl alcohol was added. The precipitate which settled out overnight was discarded; and the alcohol in the supernatant fluid was removed by distillation *in vacuo*. This material was then dissolved in 60 liters of tap water; the pH was adjusted to 7.4; and the salt mixture was added, bringing the final pH to 7.8. It was then filtered through Chamberland filters No. L 5 and inoculated immediately by adding 100 cc. of young actively growing culture to each 4 liter flask. The growth was always heavy. After overnight incubation, the organisms were killed by adding either formalin or acetic acid to a final concentration of 2 per cent. The microorganisms were removed by centrifugation in a Sharples supercentrifuge; and the clear supernatant fluid was concentrated *in vacuo* to one-tenth the original volume. To this concentrate were added 0.1 volume of glacial acetic acid, 500 gm. of sodium acetate, and 1.25 volumes of ethyl alcohol. The precipitate which formed was redissolved in 2 liters of water containing acetic acid and sodium acetate as before, and again precipitated with 1.25

TABLE I
Analyses of Carbohydrate Capsular Preparations from Group A Hemolytic Streptococci

| Lot No. | Strain | Type | Specific rotation | Total N | Total C | Acetyl | Ash |
|---------|--------|------|-------------------|-----------------|-----------------|-----------------|-----------------|
| | | | | <i>per cent</i> | <i>per cent</i> | <i>per cent</i> | <i>per cent</i> |
| 90 | S23 | 14 | -60.2 | 3.30 | 42.8 | 11.50 | 1.80 |
| 91 | S43 | 6 | -61.7 | 3.55 | 42.7 | 12.12 | 1.78 |
| 92 | S23 | 14 | -66.1 | 3.16 | 44.7 | 10.70 | 1.58 |

volumes of ethyl alcohol. The precipitate, already fairly free of protein, was again dissolved in water and shaken with chloroform and butyl alcohol (11) until a precipitate no longer formed at the interface. After a third precipitation with alcohol, the polysaccharide was dissolved in water and dialyzed 1 or 2 days against distilled water. The fourth and final precipitation was in 10 volumes of cold acetone, after adding a drop of HCl to the carbohydrate solution. This precipitate was washed in acetone and dried *in vacuo*. The final yield, at best about 120 mg. per liter of original culture, varied considerably among the different microorganisms used.

In Table I are shown certain chemical data on preparations made in the above manner with type 6 and type 14 group A hemolytic streptococci.¹ From this table it can readily be seen that the several products were fairly uniform. Furthermore, these data correspond fairly well with some of those obtained by Meyer and his collaborators in analyzing similar carbohydrates from mammalian sources (3-5). These two types of group A

¹ The type 6 strain used was S43 and the type 14 strain was S23. Both were obtained originally from the 1918 streptococcus epidemic in Texas and both had been rendered virulent for mice by repeated mouse passage.

streptococci also yielded essentially similar products. In none of the preparations was there more than a trace of blood group A substance. We were unable to remove the ash present in our preparations, either by repeated precipitation with alcohol and acetone or by prolonged dialysis against distilled water.

All of our immunological studies on the above preparations have confirmed the work of Kendall, Heidelberger, and Dawson (1), and of Seastone (2), who were unable to find any circulating antibodies in rabbits after the injection of group A and group C streptococcal vaccines. We have tried many varieties of group A streptococcal vaccines, and have employed many different strains. The vaccines were killed by heat, acetic acid, and formalin, respectively; and both young and old cultures were used. None of these vaccines, nor the prolonged injection of living cultures intravenously, ever gave rise to sera in which even a trace of precipitins for the capsular polysaccharide occurred. It seems therefore that Loewenthal (12), who has reported obtaining capsular antibodies in rabbits, was probably dealing with some impurity in his carbohydrate preparations, possibly blood group A substance.

Effect of Specific Enzymes on the Capsular Polysaccharide

In order to carry out certain *in vivo* tests on capsules, it was necessary to have a potent enzyme non-toxic for experimental animals, but still capable of splitting the capsular carbohydrate. From pneumococci, streptococci, *Bacillus welchii* (4, 7), and from soil bacilli (13), carbohydrate-splitting enzymes have been isolated. Recently, Chain and Duthie (8) described the presence of a similar enzyme in bovine testicular extract and they believe that this enzyme may be the same as the spreading factor of Duran-Reynals. Since a watery extract of leech heads has been described as the most potent source of this spreading factor (14), we decided to test the potency of such an extract for carbohydrate-splitting enzyme.² Leech extract proved to be very potent in this respect; and furthermore, this particular enzymatic activity was confined entirely to extracts of those portions of the leech containing the salivary glands.

Although such preparations contain a potent enzyme, it was necessary to eliminate the hirudin which occurs in abundance in leech extract. If the hirudin were not removed, animals treated with the extract bled easily following injection and often succumbed to a fatal hemorrhage from a simple hypodermic needle puncture. Since there exist no definite data on the

² Recently both Claude (15) and Meyer, Hobby, Chaffee, and Dawson (16) have reported the presence of a mucolytic enzyme in leech extract.

chemical nature of hirudin, we assumed that it might be similar chemically to heparin, *i.e.*, a high molecular weight polysulfuric acid ester of a polysaccharide, and hence we used as a means of getting rid of the leech anticoagulant two substances which are known to precipitate heparin. With the first of these precipitants, clupein (17), it was possible to remove about 85 per cent of the hirudin present in the leech extract; while with the second, toluidine blue (18), all but traces of the remaining 15 per cent were precipitated and removed. Both substances were used in preparing the leech extract described below because clupein removes other non-enzymatic materials from the extract besides hirudin, while toluidine blue removes the hirudin more completely.

The method of preparing leech extract began essentially as described by Claude (14): The heads of 48 leeches were cut off just behind the salivary glands, minced with scissors and ground with sand. Following this, they were extracted several times with a total of 50 cc. of water. To this solution was added about 50 mg. of clupein; and the heavy precipitate thus formed was removed and discarded. At this point the addition of more clupein did not cause further precipitation, although only 85 per cent of the hirudin had been removed. 50 mg. of toluidine blue was then added and the resulting small precipitate was removed by centrifugation. The excess of toluidine blue was removed by dialysis against distilled water. During this dialysis another precipitate formed which was also discarded. The final water-clear preparation was only slightly less potent in enzyme than the original extract. It was non-toxic when given intraperitoneally to mice, and also contained the spreading factor. There was usually about 0.5 mg. of solid material per cc. of solution.

When this leech extract was incubated with the capsular polysaccharide under suitable conditions, reducing groups were released. The enzyme was active against the similar polysaccharide of the umbilical cord and the carbohydrate obtained from a pleural tumor (3, 5).³ It was inactive against other polysaccharides tried such as chondroitin sulfuric acid and blood group A substance. The optimum activity was obtained between pH 5.0 and 6.0, although it was active from pH 4.5 to 9.0. It was completely inactivated by heating at 50°C. for one hour.

One peculiarity of this enzyme is that when added to its substrate, even in considerable excess, the final yield of reducing sugar (calculated as glucose) was only 45 to 52 per cent, whereas the theoretical yield should have been 90 per cent, assuming that the capsular polysaccharide is made up of equivalent amounts of N-acetyl glucosamine and glucuronic acid, and that the polysaccharide was completely split to these derivatives. Most of the previously described enzymes have split the substrate to the

³ These preparations were kindly given to us by Dr. M. H. Dawson.

point of yielding reducing sugar values close to the theoretical 90 per cent level. This may mean that the enzyme in leech extract is able to split only one of the carbohydrate linkages and the end product is then a disaccharide, while other enzymes or enzyme combinations split the polysaccharide down to monosaccharide derivatives. Table II shows typical activity figures for the enzyme used in various concentrations against the same amount of streptococcus capsular polysaccharide. It is clear that even when the enzyme was present in excess, the end point remained around 50 per cent.

TABLE II

Per Cent of Reducing Groups Released from Streptococcus Capsular Carbohydrate by Leech Extract

| Time | Concentration of leech extract | | | | | |
|-------------|--------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| | 0.06 per cent | 0.03 per cent | 0.015 per cent | 0.007 per cent | 0.003 per cent | 0.0015 per cent |
| <i>hrs.</i> | <i>per cent</i> | <i>per cent</i> | <i>per cent</i> | <i>per cent</i> | <i>per cent</i> | <i>per cent</i> |
| 1 | 32.2 | 23.5 | 12.7 | 7.5 | 2.5 | 1.0 |
| 3 | 42.3 | 43.0 | 36.5 | 29.2 | 18.0 | 13.0 |
| 7 | 46.5 | 47.2 | 40.0 | 32.0 | 31.5 | 23.3 |
| 24 | 50.5 | 52.5 | 47.7 | 48.7 | 47.7 | 39.5 |

The per cent concentration of leech extract is expressed in terms of dry weight of the material in the extract used. Hanes' modification of the Hagedorn-Jensen method was used for determination of reducing groups which are expressed in terms of glucose. Corrections have been made for the proper blanks in each case. The initial carbohydrate concentration in each tube was 0.1 per cent.

Effect of Leech Extract Enzyme on Streptococcus Capsules

Many authors have demonstrated the presence of capsules on group A streptococci (19, 20). One of the most satisfactory methods has been that of Seastone, who grew streptococci in high concentrations of serum. One can also demonstrate capsules surrounding microorganisms grown on a moist sealed blood agar plate. Although they are usually very difficult to detect on cocci grown in ordinary fluid media, it was discovered in the course of another investigation (21) that group A streptococci often have excellent capsules when grown in Todd-Hewitt filtered broth. In such media, the capsules are most readily made visible by the use of moist India ink preparations, in which they stand out sharply against a dark granular background. In Todd-Hewitt broth cultures of group A streptococci, the capsules appear only when the microorganisms are growing vigorously, and they are largest when multiplication of the microorganisms is most rapid. With the slowing of growth, the capsules decrease in size and shortly disappear, probably by diffusion into the medium. Likewise streptococci

virulent for mice are well encapsulated, while growing in the peritoneal cavity of the mouse. On the other hand, many strains of group A streptococci, especially those cultured long in the laboratory, are very poorly encapsulated either *in vivo* or *in vitro*. However, glossy strains as well as matt strains may be equally well encapsulated.

Similar, but much more striking and very much larger, capsules may be found on mouse virulent and guinea pig virulent group C streptococci, grown either *in vitro* or *in vivo*. The group C strains which cause non-lethal endemic infection in guinea pigs, usually have small capsules, comparable in size to those of virulent group A strains, while group C strains from human beings frequently have no demonstrable capsules at all. The capsules of the mouse virulent group C streptococci⁴ seem much more stable and last much longer in fluid culture than those of the group A strains.

If, to a drop of culture of encapsulated streptococci of either group A or group C, one adds a drop of leech extract just before making an India ink preparation, the capsules disappear almost instantly. The demonstration works equally well with encapsulated microorganisms from mouse peritoneal washings, and is most striking with the virulent group C strain (D181) since the capsules are so large. The lytic effect on the capsule was also demonstrable *in vivo* with streptococci of either group.

Two mice were each inoculated intraperitoneally with 0.5 cc. of culture. One-half hour later, one mouse was injected intraperitoneally with 0.1 cc. of leech extract. At 30 minute intervals exudate was removed from each animal and mixed with India ink. In the untreated mouse, the microorganisms remained fully capsulated, while in the one treated with enzyme, they were devoid of capsules for 2 to 4 hours, after which time they suddenly became encapsulated (see Figs. 1, 2, and 3). Another intraperitoneal injection of leech extract again removed them.

Effect of Leech Extract Enzyme on Streptococcal Infections

Having determined that the enzyme in leech extract was capable of acting on the capsules *in vivo*, the next experiment was to test the effect of the enzyme on the course of an infection. In Tables III and IV are recorded the results with two group A strains, D58⁵ and S23. Both strains were virulent for mice and both had good capsules. The mice, divided into several groups, were inoculated intraperitoneally with the indicated dilution of culture; and then one-half of each group received 0.1 cc. of purified

⁴ The mouse virulent group C strain referred to here and used throughout this paper was obtained from Dr. Seastone and is referred to in his paper (2) as strain No. 4. Our designation for this strain is D181.

⁵ D58 is a mouse virulent strain of a type 3 group A hemolytic streptococcus, obtained by Colebrook from puerperal sepsis, and is called by him strain "Richards."

leech extract diluted to 0.5 cc. in saline. The extract was given intraperitoneally a few minutes following the inoculation, then every 8 hours

TABLE III

Protective Action of Leech Extract in Mice Infected with Group A Streptococcus, Type 14 (Strain S23)

| Mouse No. | Treated with leech extract | | | |
|-----------|----------------------------|----------------------|----------------------|----------------------|
| | Dilution of culture | | | |
| | 10 ⁻⁸ cc. | 10 ⁻⁶ cc. | 10 ⁻⁷ cc. | 10 ⁻⁸ cc. |
| 1 | D1 | D1 | D2 | S |
| 2 | D1 | D1 | D3 | S |
| 3 | D1 | D1 | D3 | S |
| 4 | D1 | D1 | S | S |
| 5 | D2 | D2 | S | S |
| 6 | D2 | D3 | S | S |
| 7 | D3 | D10 | S | S |
| 8 | D3 | D10 | S | S |
| 9 | D4 | S | S | S |
| 10 | D5 | S | S | S |

| Mouse No. | Virulence controls | | | |
|-----------|----------------------|----------------------|----------------------|----------------------|
| | Dilution of culture | | | |
| | 10 ⁻⁸ cc. | 10 ⁻⁶ cc. | 10 ⁻⁷ cc. | 10 ⁻⁸ cc. |
| 1 | D1 | D1 | D1 | D1 |
| 2 | D1 | D1 | D1 | D10 |
| 3 | D1 | D1 | D1 | S |
| 4 | D1 | D1 | S | S |
| 5 | D3 | D1 | S | S |
| 6 | D3 | D2 | S | S |
| 7 | D3 | D2 | S | S |
| 8 | D10 | D2 | S | S |
| 9 | S | D3 | S | S |
| 10 | S | S | S | S |

All mice were inoculated first with respective dilutions of culture. Within one-half hour after infection, all treated mice received 0.1 cc. of leech extract intraperitoneally. The treated mice were then given 0.1 cc. of leech extract at 8 hour intervals for 48 hours, and at 12 hour intervals during the 3rd and 4th days. The experiments shown in Tables IV and V were done in a similar way.

D, with a number, indicates death within that number of days. S means survival throughout time of experiment, 10 days.

for 2 days, and every 12 hours on the 3rd and 4th days. Under the conditions of these experiments, there was only suggestive evidence that the enzyme had a slight influence on the lethal action of strain S23, in that the groups of mice inoculated with 10⁻⁸ and 10⁻⁷ cc. of culture survived

longer when treated with the leech extract, even though there was no significant difference in the number of survivors. This tendency to delayed death was distinctly more marked in mice inoculated with strain D58.

TABLE IV
Protective Action of Leech Extract in Mice Infected with Group A Streptococcus, Type 3 (D58, Strain "Richards")

| Mouse No. | Treated with leech extract | | | |
|-----------|----------------------------|----------------------|----------------------|----------------------|
| | Dilution of culture | | | |
| | 10 ⁻⁶ cc. | 10 ⁻⁶ cc. | 10 ⁻⁷ cc. | 10 ⁻⁸ cc. |
| 1 | D1 | D1 | D2 | D1 |
| 2 | D2 | D1 | D2 | D1 |
| 3 | D2 | D1 | D4 | D2 |
| 4 | D2 | D1 | D4 | D4 |
| 5 | D3 | D2 | S | D7 |
| 6 | D3 | D3 | S | S |
| 7 | D4 | D3 | S | S |
| 8 | D4 | D3 | S | S |
| 9 | S | D3 | S | S |
| 10 | S | S | S | S |

| Mouse No. | Virulence controls | | | |
|-----------|----------------------|----------------------|----------------------|----------------------|
| | Dilution of culture | | | |
| | 10 ⁻⁶ cc. | 10 ⁻⁶ cc. | 10 ⁻⁷ cc. | 10 ⁻⁸ cc. |
| 1 | D1 | D1 | D1 | D1 |
| 2 | D1 | D1 | D1 | D1 |
| 3 | D1 | D1 | D1 | D1 |
| 4 | D1 | D1 | S | D1 |
| 5 | D1 | D1 | S | S |
| 6 | D1 | D1 | S | S |
| 7 | D1 | D1 | S | S |
| 8 | D1 | S | S | S |
| 9 | D1 | S | S | S |
| 10 | S | S | S | S |

See note under Table III.

When, on the other hand, a similar experiment (Table V) was made with the virulent group C streptococcus, strain D181, a marked protective influence of the leech extract was demonstrated. The enzyme protected the mice completely up to and including 10,000 M.L.D.'s, which was the maximum employed. When still another group of controls was treated with the same leech extract, which had been inactivated by heating at 50°C. for 40 minutes, it no longer offered protection, showing that the protective

factor has about the same heat lability as the carbohydrate-splitting enzyme. If the animals receiving the active enzyme were not treated intensively following infection, the amount of protection was much less; and even those treated effectively for 4 days occasionally suffered recurrences and died 4 to 8 days after the treatment was discontinued.

TABLE V
Protective Action of Leech Extract in Mice Infected with Group C Streptococcus Strain (D181)

| Treated with leech extract | | | | | |
|----------------------------|----------------------|----------------------|----------------------|----------------------|----------------------|
| Dilution of culture | | | | | |
| 10 ⁻³ cc. | 10 ⁻³ cc. | 10 ⁻⁴ cc. | 10 ⁻⁵ cc. | 10 ⁻⁶ cc. | 10 ⁻⁷ cc. |
| S | S | S | S | S | S |
| S | S | S | S | S | S |
| S | S | S | S | S | S |
| S | S | S | S | S | S |

| Treated with leech extract heated (56° 40 minutes) | | | | | |
|----------------------------------------------------|----------------------|----------------------|----------------------|----------------------|----------------------|
| Dilution of culture | | | | | |
| 10 ⁻³ cc. | 10 ⁻³ cc. | 10 ⁻⁴ cc. | 10 ⁻⁵ cc. | 10 ⁻⁶ cc. | 10 ⁻⁷ cc. |
| D1 | D1 | D2 | D2 | D2 | S |
| D1 | D1 | D2 | D2 | D2 | S |
| D1 | D2 | D2 | D2 | D2 | S |
| D2 | D2 | D2 | D4 | S | S |

| Virulence controls | | | | | |
|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|
| Dilution of culture | | | | | |
| 10 ⁻³ cc. | 10 ⁻³ cc. | 10 ⁻⁴ cc. | 10 ⁻⁵ cc. | 10 ⁻⁶ cc. | 10 ⁻⁷ cc. |
| D1 | D1 | D2 | D2 | D2 | S |
| D1 | D1 | D2 | D2 | D2 | S |
| D1 | D2 | D2 | D2 | D2 | S |
| D2 | D2 | D2 | D3 | D2 | S |

In this experiment the mice were treated for 4 days and the experiment was terminated on the 6th day. See note under Table III.

A similar experiment was carried out in guinea pigs, since they are the natural hosts of this infectious agent. In this experiment, summarized in Table VI, the guinea pigs were infected intraperitoneally with graded doses of the same group C strain used in mice. The controls died in 1 to 6 days, all having peritonitis and blood stream invasion. The treated guinea pigs were given 0.3 cc. of leech extract intraperitoneally every 8 hours for 3 days and every 12 hours for 3 additional days, beginning a few minutes after

inoculation. All the treated guinea pigs were sacrificed at the end of a week; and cultures of the blood, abdominal abscesses, and peritoneal surface were made. In no case was there any peritonitis or peritoneal abscess. The four guinea pigs receiving the highest doses of inoculum had subcutaneous abscesses and varying degrees of bacteremia, while in the remaining two, no evidence of infection was found. Since the control animals all died of peritonitis, while the treated ones had none, it seems plain that the extract was able to control this phase of the infection. Those guinea

TABLE VI

Protective Action of Leech Extract in Guinea Pigs Infected with Group C Streptococcus, Strain D181

| Infecting dose | | | | | | |
|-----------------------------------------------------------------------------------------------------------------------------------------|------|------|------|------|------------|------------|
| | | | | | | |
| 10 ⁻¹ cc. 10 ⁻² cc. 10 ⁻³ cc. 10 ⁻⁴ cc. 10 ⁻⁵ cc. 10 ⁻⁶ cc. | | | | | | |
| Treated | | | | | | |
| | S | S | S | S | S | S |
| Blood culture | + | +++ | ++++ | ++ | - | - |
| Peritoneal culture | - | - | - | - | - | - |
| Skin abscess culture | ++++ | ++++ | ++++ | ++++ | No abscess | No abscess |
| Controls | | | | | | |
| | D1 | D6 | D2 | D2 | D5 | D2 |
| Blood culture | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ |
| Peritoneal culture | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ |

Treatment was started within 30 minutes after the infecting dose had been given. Treated guinea pigs received 0.3 cc. of leech extract, intraperitoneally, every 8 hours for 72 hours, and every 12 hours thereafter, until the experiment was terminated at one week. See Table III for further explanation.

pigs that had the abscesses and blood stream infection, probably would have eventually succumbed even had the treatment been continued. Doubtless, these animals received a small subcutaneous infection along the tract of the needle used for intraperitoneal inoculation; and it seems likely that the enzyme preparation had little or no effect on the infection in this site, and from this abscess the organisms were able to multiply and invade the blood stream.

DISCUSSION

The capsule of group A and group C streptococci does not seem to be an organized structure in the same sense that a cell wall is a structure. Rather it seems to be merely an accretion around the microorganism of a slowly

diffusible substance, which during the active phase of bacterial growth is produced faster than it diffuses away. The capacity to produce this capsular substance varies enormously among the different strains, but is always well developed in mouse virulent strains of both groups of streptococci; and in some mouse virulent group C strains its production is very marked.

Seastone (2) has presented evidence indicating that the large capsules of virulent group C strains are responsible in part for their increased virulence. In his infected colony of guinea pigs, the endemic microorganism was a group C streptococcus which had the power of infecting the animals without killing them; and the infection remained localized in lymph nodes. When, however, a virulent group C strain appeared in the colony, probably by variation of the endemic strain, it killed large numbers of the guinea pig population with an acute disease. This new strain was always much more heavily capsulated than the endemic strain. Seastone tried in vain to protect animals against this virulent infection, both by active and passive immunization; but only in those animals having chronic infection with the endemic strain was any protection observed, and even here was very slight.

In our experiments with group C streptococcal infections of both mice and guinea pigs, we obtained good protection by the use of an enzyme which would effectively remove these large capsules, and it seems at least possible that the saccharolytic and protective agent are the same. This offers further substantial evidence that the capsule of these group C streptococci is an important factor in making these strains virulent for mice and guinea pigs. This *in vivo* demonstration of the effect of the enzyme on the capsule was limited in these experiments to peritoneal infections, where the infectious process was so circumscribed that the microorganisms were readily accessible to the therapeutic agent. The presence of the spreading factor in these leech extracts made it impossible to show a similar effect on subcutaneous infections, since the spreading factor, if applied there, would probably have extended the local area of infection. The fact that repeated intraperitoneal administration of the enzyme is necessary for protection, and that a demonstrable decapsulating effect lasts only a few hours is probably due to inactivation of the active principle, possibly by exhaustion on a similar substrate in the host.

With group A streptococci, the story is somewhat different. In this group we have never encountered a strain which has capsules comparable in size to the virulent group C strain. While there is some parallelism between the appearance of mucoid colonies and virulence of many strains of group A, nevertheless, the importance of another factor associated with virulence, the M substance, has been amply demonstrated (22). The M

substance is always present in mouse virulent strains; and sera containing sufficient anti-M antibody has a marked protective effect against group A streptococcus infection of mice. From the results of the present experiments, it would seem that the capsule plays a minor rôle in the virulence and invasiveness of these particular microorganisms. Here again it seems unwise to generalize from the very special type of infection where bacteria are put into the peritoneal cavity of the mouse and the decapsulating enzyme is applied at the same site, to instances where the infection is in other tissues in which it is impossible to bring the enzyme so closely into contact with the microorganism.

SUMMARY

1. Confirming the observations of other experimenters, it has been found that group A hemolytic streptococci produce a capsule containing a polysaccharide which is similar to, if not identical with, certain high molecular weight sugars found in the mammalian body.

2. Leech extract possesses a powerful enzyme capable of splitting one of the linkages in this polysaccharide and of decapsulating group A and group C hemolytic streptococci *in vitro* and *in vivo*.

3. Mice and guinea pigs can be protected from intraperitoneal infection with a virulent group C streptococcus by the intraperitoneal administration of leech extract. In contrast there is little protective action of leech extract in mice infected with group A hemolytic streptococci.

4. The protective effect of leech extract against streptococcal group C infection is probably due to the removal of the capsule *in vivo*.

5. The capsule of mouse virulent group C streptococci plays a major rôle in the virulence of that microorganism, while the capsule of certain mouse virulent group A streptococci plays little, if any, rôle in virulence, at least when the infection is intraperitoneal in the mouse.

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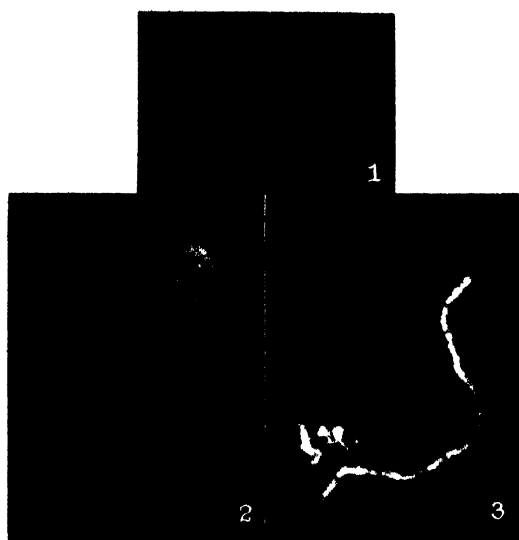
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EXPLANATION OF PLATE 26

FIG. 1. Capsule on group A hemolytic streptococci (strain S23), grown in mouse peritoneum. India ink preparation. $\times 1,000$.

FIG. 2. Capsule on group C hemolytic streptococci (strain D181), grown in mouse peritoneum. India ink preparation. $\times 1,000$.

FIG. 3. Decapsulated group C hemolytic streptococci (strain D181), grown in mouse peritoneum, and withdrawn 30 minutes after intraperitoneal injection of leech extract. India ink preparation. $\times 1,000$.



(Hirst Streptococcal capsular splitting enzyme)

IDENTIFICATION OF THE VIRUS OF LYMPHOCYTIC CHORIOMENINGITIS

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A relatively simple and rapid method of identifying the virus of lymphocytic choriomeningitis is of importance not only for special workers in the virus field but also for those called upon in a routine manner to isolate and identify etiological agents of human diseases. The availability of such a technique is especially desirable because this virus has been isolated from several species of animals used in experimental work, e.g., from white mice by Traub (1935, 1936), Findlay, Alcock and Stern (1936), Lépine and Sautter (1936), and Kasahara, Hamano and Yamada (1939), and from gray house mice by Armstrong and Sweet (1939) and Armstrong, Wallace and Ross (1940); from monkeys by Armstrong and Wooley (1935) and Coggeshall (1939); from guinea pigs by Kasahara and coworkers (1939); and from dogs by Dalldorf (1939) and Howitt (1939-40). Not infrequently the virus has been found in normal-appearing animals, and for that reason Andrewes (1939) has placed it in the group of "indigenous viruses." The capacity to produce an inapparent infection probably accounts for the relative ease with which the virus of lymphocytic choriomeningitis has been picked up as a contaminant during serial transmission of other viruses. For example, it has been encountered by Dalldorf (1939) during passage in dogs of the virus of canine distemper and by Casals-Ariet and Webster (1940) in serial tissue cultures of rabic virus. In the latter instance the workers believe that the contaminating agent was introduced by way of the monkey serum rather than the mouse embryo tissue used in the culture medium.

The classical technique for the identification of a virus, namely,

by means of cross-immunity and neutralization tests, is expensive and time-consuming. An abbreviated form of this method, which consists of inoculating the unknown agent into animals immune to known viruses, is available only to those workers who have on hand a constant supply of such immune animals. The complement fixation obtained with materials from animals with lymphocytic choriomeningitis (literature reviewed by Smadel and Wall (1940)) seemed to offer a satisfactory means for diagnosing this disease without recourse to more laborious techniques. The present report deals with the identification of the agent of lymphocytic choriomeningitis by the use of complement fixation; this is accomplished by the demonstration in infected animals of the specific soluble antigen associated with the disease and the subsequent development of anti-soluble-substance antibodies in animals that survive infection.

METHODS

Complement-fixation tests

Details of the technique of the test have been previously described (Smadel, Baird and Wall, 1939). Briefly, the procedure was as follows: 0.2 ml. of falling dilutions of the materials to be tested were mixed with equal amounts of hyperimmune serum (5 units) or of standardized antigenic solution (5 units), depending on whether antigen or antibody was being identified. Then 2 units of complement were added to each tube, and the mixtures were allowed to stand overnight at 3°C. The following morning 0.5 ml. of a 5 per cent suspension of washed sheep erythrocytes and 0.2 ml. of anti-sheep-cell amboceptor (2 units of hemolysin) were added to each tube. A final reading of the amount of hemolysis was made after the tubes had been in a water-bath at 37°C. for 30 minutes. Suitable anticomplementary and hemolytic controls, as well as one with a hyperimmune guinea pig serum of known titer, were included in each set of tests.

Antigen

Stock solutions of complement-fixing antigen were prepared according to the routine used in this laboratory (Smadel, Baird and Wall, 1939). A 10 per cent suspension prepared from 5 to

30 spleens taken from guinea pigs infected with the W.E. strain of virus was freed of large particles of tissue by ordinary centrifugation; after the supernatant fluid was spun in the ultracentrifuge (Bauer and Pickels, 1936) at 30,000 R.P.M. for 20 minutes, it was filtered through a Seitz pad. Then merthiolate¹ was added to a concentration of 1:10,000 in order to maintain bacteriological sterility during storage in the cold for periods of time up to 6 months. Antigenic solutions prepared in such a manner contained little or no virus and as a rule had a complement-fixing titer of 1:16 in the presence of hyperimmune guinea pig serum. At times when new or unidentified strains of viruses were being studied extracts were prepared from a single infected guinea pig spleen; when such small amounts of material were used Seitz filtration and in some instances ultracentrifugation were omitted. Ten per cent suspensions of infected spleens which had been cleared of gross particles by means of centrifugation at 3000 R.P.M. for 20 minutes were rarely anticomplementary and were employed successfully in the work reported in this communication. Such crude fresh preparations have a limited use, however, as has been pointed out elsewhere (Smadel, Wall and Baird, 1940).

Antiserum

For the detection of antibodies in guinea pigs convalescent from infection with a particular strain of virus, serum was obtained by cardiac puncture while the animals were under ether anesthesia. Properly diluted hyperimmune serum from either guinea pigs or from mice was used in tests for the presence of soluble antigen in splenic extracts. It has recently been found that the injection of formolized soluble antigen into resting hyperimmune guinea pigs provides the simplest means so far available for obtaining complement-fixing serum of consistently high titer (Smadel and Wall, 1940).

RESULTS

It has previously been demonstrated by means of complement fixation that animals infected with any one of the 4 strains

¹ Product of Eli Lilly and Company.

of lymphocytic choriomeningitis virus maintained in this laboratory possess in their tissues a soluble antigen which is separable from the virus and is specific for the disease; moreover, no evidence of type specificity was encountered in the antigens associated with these strains (Smadel, Baird and Wall, 1939). During the last 1½ years, we have examined by means of complement fixation materials from animals infected with 9 additional strains of the virus. In each instance the soluble antigen was detected in the tissues of infected animals. Furthermore, antibodies for the soluble antigen were manifest in the serum of surviving guinea pigs. Identification of the virus was thus established in studies on animals of the first passage; results obtained by complement fixation were verified by cross-immunity tests. The classical method was used with several of the 9 strains, but with the remainder it seemed sufficient to show that guinea pigs or mice known to be immune to the W. E. strain of virus were not susceptible to infection with the agent in question.

The technique of rapid identification of the virus of lymphocytic choriomeningitis is illustrated by a summary of the work on one typical strain, S.M. Portions of a bacteriologically sterile mixture of cerebrospinal fluid and blood, taken 6 days after onset of illness from S.M. who was suspected of having lymphocytic choriomeningitis, were inoculated by intracerebral, intraperitoneal and subcutaneous routes into 2 guinea pigs (G,1-1 and G,1-2) and intracerebrally into 6 mice. Both guinea pigs developed fever on the 4th day after inoculation. On the 5th day one of the animals (G,1-1) was sacrificed by bleeding from the heart while under ether anesthesia, and 1 ml. of its heparinized blood was immediately inoculated subcutaneously into each of 2 normal guinea pigs (G,2-1 and G,2-2) and into a guinea pig (G,2-3) known to be immune to lymphocytic choriomeningitis. The brain of G,1-1 was removed aseptically and stored in the cold as a source of this strain of virus. A 1:4 dilution of an extract prepared from the spleen of this animal fixed complement in the presence of a known hyperimmune serum. The other guinea pig of the first passage, G,1-2, had fever for 8 days during which time it appeared ill and lost weight, but by the end of 2

weeks it had begun to improve. Serum taken from this animal 3 weeks after inoculation contained anti-soluble-substance antibodies in a titer of 1:16. Finally, this pig was subsequently found to be immune to a test dose² of the virus of lymphocytic choriomeningitis. The 2 guinea pigs (G,2-1 and G,2-2) inoculated with heparinized blood from the first passage animals died on the 16th and 17th days, respectively, after exhibiting typical signs of the disease in this host, i.e., fever, conjunctivitis, salivation, somnolence, loss of weight, labored breathing and prostration. The hyperimmune pig, G,2-3, which had been inoculated with a portion of the same infectious blood remained well. All of the 6 mice which received part of the mixture of cerebrospinal fluid and blood from S.M. died on the 6th day after inoculation, and a 1:16 dilution of an extract of their pooled spleens fixed complement in the presence of hyperimmune mouse serum.

In the manner described above, the specific soluble antigen of lymphocytic choriomeningitis was detected in guinea pigs and in mice on the 5th and 6th days, respectively, after inoculation with material from the acutely ill patient, S.M. The nature of the infectious agent was thus established before the patient was discharged from the hospital and approximately 2 weeks before complement-fixing antibodies for the soluble antigen of lymphocytic choriomeningitis were detectable either in the patient's serum (Smadel and Wall, 1940) or in the serum of a surviving guinea pig of the first passage. Identification of the agent by cross-immunity tests was comparatively rapid in this instance; nevertheless, data from these tests were not available for tentative classification until 2 weeks after receipt of material and were not complete until a month later.

Eight additional infectious agents were shown to be strains of lymphocytic choriomeningitis virus by the demonstration of

² A test dose of virus is prepared as follows: A mixture of equal parts of normal guinea pig serum and of a 10^{-2} dilution of guinea pig brain infected with the W.E. strain of virus is held at 37°C. for 1 hour. Then 0.5 ml. of the mixture is inoculated subcutaneously into a guinea pig or 0.03 ml. is given intracerebrally into a mouse; such an inoculum contains from 100 to 1000 M.L.D. of virus. Animals receiving the infectious material are observed for 18 days and their temperatures are recorded daily during this period.

the presence of the soluble antigen specific for this agent in extracts of spleens prepared from febrile guinea pigs which had

TABLE 1

Demonstration of the presence of soluble antigen and anti-soluble-substance antibodies in guinea pigs infected with strains of lymphocytic choriomeningitis virus

| STRAIN | ORIGINAL MATERIAL INJECTED INTO GUINEA PIGS | PRESENCE OF SOLUBLE ANTIGEN IN SPLEENS OF INFECTED ANIMALS | DAY OF SPLENIC HARVEST, 1st PASSAGE | DEVELOPMENT OF C-F ANTIBODIES IN SERUM OF RECOVERED ANIMALS | CROSS-IMMUNITY WITH W.E. STRAIN OF VIRUS |
|-------------|---------------------------------------------|------------------------------------------------------------|-------------------------------------|-------------------------------------------------------------|------------------------------------------|
| S.M. (1) | Human spinal fluid and blood | + | 5th | + | + |
| K.P. (2) | Human spinal fluid and blood | + | 9th | + | + |
| M-2 (3) | Guinea pig brain | + | 7th | + | + |
| A.S. (4) | Mouse brain | + | 8th | + | + |
| McC. (5) | Human spinal fluid | + | 4th | + | + |
| J.P. (6) | Guinea pig brain | + | 6th | -* | + |
| J.L. (7) | Human spinal fluid | + | 11th | + | + |
| S. (8) | Mouse brain | + | 8th | + | + |
| II S.F. (9) | Mouse brain | + | 12th | + | + |

* Animals died before antibodies developed.

Strains isolated from human beings with lymphocytic choriomeningitis:

- (1) S.M., patient of Dr. J. Schneider, Lincoln Hospital, New York.
- (2) K.P., patient of Dr. J. S. Vanneman, Princeton Hospital, Princeton, New Jersey.
- (3) M-2, isolated from patient and identified by Dr. H. L. Hodes, Sydenham Hospital, Baltimore, Maryland.
- (4) A.S., isolated from patient by Dr. G. O. Broun, Firmin Desloge Hospital, St. Louis, Missouri.

Strains isolated from animals inoculated with human material:

- (5) McC., patient in Rockefeller Hospital, New York.
- (6) J.P., isolated by Dr. S. O. Levinson from a guinea pig inoculated with spinal fluid from a suspected case of lymphocytic choriomeningitis, Samuel Deutsch Convalescent Serum Center, Michael Reese Hospital, Chicago, Illinois.
- (7) J.L., patient of Dr. Donald D. Parker, Presbyterian Hospital, New York.

Strains isolated from apparently normal animals:

- (8) S., isolated from a "normal" mouse in the laboratories of the International Health Division of The Rockefeller Foundation.
- (9) II S.F., isolated during serial tissue culture of rabic virus, probably from monkey serum (Casals-Ariet and Webster, 1940).

been inoculated with the original materials received in our laboratory. Moreover, the spleens of mice which received 3 of the

strains were found to contain the soluble antigen. In addition, anti-soluble-substance antibodies were demonstrable in animals that recovered from infection produced by 8 of the 9 strains of virus; animals inoculated with strain J.P. invariably died before antibodies developed. In each instance subsequent cross-immunity tests confirmed the identification based on data derived from *in vitro* reactions. Results obtained in studies of the 9 strains of virus are summarized in table 1.

The 9 strains of virus investigated in this work varied considerably in their pathogenicity for guinea pigs. Three of them, J.P., McC. and S., were so highly virulent that the survival of an animal, even when inoculated with only a few infective doses, occurred rarely or not at all. On the other hand, strain II S.F. induced such a moderate disease in guinea pigs that they displayed only a slight fever for a few days. Inasmuch as the amount of antigen is small (titer not above 1:2) in spleens of guinea pigs infected with a mild strain such as W.W.S. (Smadel, Baird and Wall, 1939) or II S.F., the interpretation of results of the complement-fixation test may be difficult. In such instances, identification of the virus rests on the detection of anti-soluble-substance antibodies in the serum of convalescent animals, or on some other type of procedure. On the other hand, the titer of soluble antigen in extracts prepared from spleens infected with a highly virulent strain, e.g., W.E. (Smadel *et. al.*, 1939) or J.P., is sufficiently high (1:16 to 1:32) for the result to be of diagnostic value.

DISCUSSION

The identification of the virus of lymphocytic choriomeningitis by means of complement fixation is an adequate, simple and inexpensive procedure. Solutions of specific soluble antigen and complement-fixing antiserum for use in the test may be kept for several months at ordinary ice box temperatures without appreciable loss of activity. Moreover, both materials when dried *in vacuo* from the frozen state and stored in the ice box remain active for longer periods of time.

The data presented in this report deal chiefly with the identi-

fication of the virus of lymphocytic choriomeningitis rather than with the significance that may be attached to the recovery of the virus from animals inoculated with supposedly infectious material. It should be emphasized that isolation of the virus from one of a number of animals inoculated with material from human beings does not constitute proof that this virus is the etiological agent of the human disease. The frequency with which apparently normal animals from certain stocks develop clinical manifestations of infection with the virus of lymphocytic choriomeningitis after intracerebral injection of sterile material (Traub, 1935, 1936, and Findlay and coworkers, 1936) should make one attach little significance to the illness of a single inoculated animal. The simultaneous illness of a number of the inoculated animals and the development of immunity in most of those surviving infection contribute to the possibility that the virus in question was isolated from the patient and not encountered fortuitously in the experimental animals. The opinion held by workers in this laboratory, which has been expressed elsewhere (Scott and Rivers, 1936), is that proof that a given human illness is caused by the virus of lymphocytic choriomeningitis rests ultimately on the demonstration of the development of antibodies in the serum of the patient. Thus, examination should show that a specimen of serum taken at the onset of the disease does not contain complement-fixing or neutralizing antibodies and that these antibodies appear in the patient's blood during convalescence. A strict adherence to these criteria may occasionally result in a falsely negative diagnosis, since MacCallum and Findlay (1939) and Howard (1939, 1940) have reported that the appearance of neutralizing antibodies in human beings may be delayed beyond the usual time of appearance—6 to 8 weeks—or fail entirely to develop. It should be pointed out that 2 strains of virus identified in our study, i.e., McC. and J.L., were recovered from 1 out of 6 and 1 out of 8 animals, respectively, inoculated with material from patients who developed neither complement-fixing nor neutralizing antibodies during convalescence; furthermore, none of the remaining animals became immune to the virus. Consequently these strains of virus were not regarded as

the cause of disease in these two individuals. Their origin is uncertain but two possible sources are obvious. In the first place, guinea pigs from our Institute-bred stock which is known to be free from lymphocytic choriomeningitis are occasionally not available; consequently, animals from commercial sources received material from one of these two patients. In the second place, the manipulation and trauma incident to the taking of daily rectal temperature in a large group of guinea pigs, some of which are suffering from experimentally induced lymphocytic choriomeningitis, offer a means of cross-infection. The foregoing experience emphasizes the importance of employing animals from stocks known to be free from infection with the virus of lymphocytic choriomeningitis and of following strict precautions of isolation in the housing and care of experimental animals.

SUMMARY

Nine strains of the virus of lymphocytic choriomeningitis were identified by means of complement fixation. The soluble antigen associated with the disease and responsible for fixation was detected in the spleens of infected guinea pigs and mice. In addition, the development of anti-soluble-substance antibodies was demonstrated in guinea pigs surviving infection with the agents. The significance which may be attached to the isolation of the virus from animals inoculated with human material is discussed.

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CONSTITUENTS OF ELEMENTARY BODIES OF VACCINIA

IV. DEMONSTRATION OF COPPER IN THE PURIFIED VIRUS

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PLATE 6

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Previous studies have indicated that preparations of elementary bodies of vaccinia can be obtained which exhibit great constancy in their immunological, physical, and chemical properties. These studies have shown that the virus is composed chiefly of protein, thymonucleic acid, lipid, and carbohydrate, which occur in constant proportions in different lots of purified virus, and in concentrations not materially different from those found in bacterial cells (1, 2). No independent metabolism of elementary bodies has yet been discovered. Parker and Smythe (3), in 1935, were unable to demonstrate the utilization of oxygen by purified elementary bodies in the presence of hexose-monophosphate and "respiratory factor;" nor could they obtain evidence of acid production under anaerobic conditions with glucose-monophosphate, bicarbonate buffer, and an extract of tissue as a source of respiratory supplement.

The obligate parasitic nature of the elementary body of vaccinia makes it unlikely that an independent metabolism, apart from the host cell, exists. It is not unreasonable to assume, however, that the elementary body possesses an incomplete metabolic system, relying in part, or even in the main, on constituents within the host cell for its completion. With this hypothesis in mind we have searched for the presence in purified virus of substances which, in bacteria and more highly organized cells, are known to participate in oxidation-reduction chains. In this paper results of our search are recorded.

EXPERIMENTAL

A detailed description of the methods used in the concentration and purification of elementary bodies of vaccinia has appeared in an earlier publication (1). Freshly prepared, active virus, dried to constant weight, was used in each experiment recorded in this study.

Attempts to Demonstrate a Cytochrome System

Respiration in aerobic organisms is known to go in part, if not chiefly, through the cytochrome system, which acts as a reversible oxidation-reduction

link between oxygen and certain enzyme systems which have become reduced in the process of the oxidation of cell substrates (4, 5). So far as it is now known, all cells which are able to use molecular oxygen contain one or more members of a group of protein porphyrins with iron in tetra-pyrrolic combination. These substances are known collectively as the cytochromes, and have been shown to participate in reversible oxidation-reduction processes within the cell (5). An enzyme, cytochrome oxidase, concerned with the rapid oxidation of reduced cytochrome, has been studied in detail by Keilin and Hartree (6). Both spectroscopic and enzymatic methods are available for the detection of cytochrome *c* (7).

Spectroscopic Examination.—In selected biological material, suitably prepared, the bands of reduced cytochrome can often be seen clearly with the aid of a microspectroscope. A search for the cytochrome system in vaccine virus was first made spectroscopically by means of a small Zeiss microspectroscope which was substituted for the eyepiece of an ordinary monocular microscope. A hanging drop preparation, containing a thick suspension of elementary bodies in 5 per cent sodium hyposulfite was prepared and examined repeatedly with a strong light source for the bands of reduced cytochrome. No absorption of any type was evident within the visible range of the spectrum. Under the same conditions the bands of reduced cytochrome in a suspension of yeast were clearly observed. The opacity of the elementary body preparation, however, made it possible that faint bands of reduced cytochrome might well have been obscured, and consequently missed by this technique. We accordingly turned to the second method, employing cytochrome *c*, cytochrome oxidase, and a variety of hydrogen donors, in an attempt to demonstrate cytochrome oxidase and cytochrome *c*, respectively, in preparations of elementary bodies of vaccinia.

Examination of Virus for Cytochrome Oxidase.—Cytochrome *c* was prepared by the method of Keilin and Hartree (8). This substance, together with paraphenylenediamine and certain other hydrogen donors, can be used to demonstrate the presence of cytochrome oxidase in suitably prepared biological materials, as shown by oxygen uptake in the Warburg apparatus. Under carefully controlled conditions, the rate of oxidation of the paraphenylenediamine in the presence of pure cytochrome *c* is proportional to the concentration of the cytochrome oxidase which is contained in the substance tested.

1000 gm. of fresh beef heart muscle were freed from fat and ligaments and ground finely in a meat chopper. The pulp was mixed with an equal volume of 0.15 N (2.5 per cent) trichloroacetic acid. The mixture was allowed to stand at room temperature for 2 hours with occasional stirring, after which the fluid was pressed out, brought to pH 7 with sodium hydroxide, and centrifuged for 10 minutes. The clear supernatant fluid was drawn off by suction, and 50 gm. of ammonium sulfate for each 100 cc. of material was added. The precipitate was filtered off and discarded. The filtrate

was next treated with an additional 5 gm. of ammonium sulfate per 100 cc. and allowed to stand overnight in the cold. The material was then filtered, and while cold there was added 1/40 volume of cold 20 per cent trichloroacetic acid. The fine red precipitate which appeared at this point in the procedure was collected by centrifugation. The precipitate was shaken with saturated ammonium sulfate and centrifuged again, after which it was transferred to a cellophane sac in a minimal amount of distilled water and dialyzed for 48 hours at 4°C. against 1 per cent sodium chloride. Finally the contents of the sac were shaken with a few drops of chloroform, filtered, and frozen and dried *in vacuo*.

An analysis performed on a sample of this material, dried to constant weight and ashed, revealed an iron content of 0.4 per cent which is close to the value given by Theorell and Åkesson for purified cytochrome *c* (9). The high activity of this material was demonstrated by oxygen uptake in the presence of paraphenylenediamine and cytochrome oxidase in the Warburg respirometer.

A sample of cytochrome *c* prepared by this technique was diluted with water until 1 cc. gave an oxygen uptake of 250 to 300 c.mm. per hour in the presence of an excess of hydroquinone and cytochrome oxidase. Freshly prepared elementary bodies, in 5 to 15 mg. lots, were suspended in 1.5 cc. of phosphate buffer, mixed with 0.5 cc. of the diluted cytochrome *c* solution, and placed in a Warburg respiration flask. In the side arm was placed 0.5 cc. of water containing 3.6 mg. of hydroquinone. The flask was attached to the manometer, cock sealed, and shaken in the water bath at 37°C. When temperature equilibrium had been reached, the hydroquinone solution was tipped into the elementary body-cytochrome *c* mixture, and the manometer read at frequent intervals for oxygen uptake. Control flasks, with cytochrome *c* and hydroquinone alone, were set up at the same time.

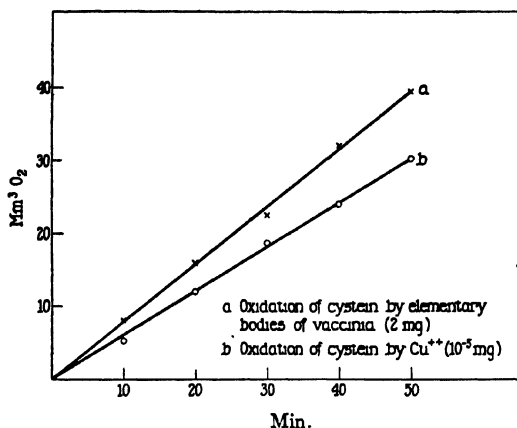
No significant oxygen uptake of the elementary body-cytochrome *c*-hydroquinone mixture, over that of the control flasks, was observed after 2 hours when the experiment was discontinued. If the virus contained cytochrome oxidase it was not possible to demonstrate it by this technique.

Examination of Virus for Cytochrome c.—A partially purified cytochrome oxidase for use in testing for cytochrome *c* was prepared from beef heart by the technique of Stotz and Hastings (10).

50 gm. of beef heart muscle were freed from fat and ligaments and ground twice in a meat chopper. The finely ground material was then placed in a bag made of two thicknesses of bandage gauze and washed in 1 liter of tap water at 40°C. for 10 minutes, with occasional squeezing. This procedure was continued through 4 washings at 40°C., alternating with 4 washings at 15°C. The mass of washed material, squeezed dry, was next placed in a mortar and ground with 60 mesh alundum in 100 cc. of $\text{M}/15 \text{ K}_2\text{HPO}_4$ until a smooth paste was obtained. The paste was allowed to stand at room temperature 30 minutes with occasional stirring. The material was then centrifuged at 2000 R.P.M. and the sediment discarded. To the supernatant, which was allowed to stand at 4°C. overnight, was added an equal volume of 0.2 molar acetate buffer, pH 4.5. The mixture was then centrifuged and the supernatant

material discarded. The precipitate was resuspended in 10 cc. of $M/15 K_2HPO_4$. 0.5 cc. of this cytochrome oxidase preparation in the Warburg manometer was sufficient to yield an uptake of 20 to 30 c.mm. of oxygen per 10 minutes in the presence of an active preparation of cytochrome *c* and hydroquinone.

Purified elementary bodies, freshly prepared, in 5 to 15 mg. lots were suspended in 1.5 cc. of phosphate buffer and mixed with 0.5 cc. of an active suspension of cytochrome oxidase in the Warburg respirometer flask. In the side arm was placed 0.5 cc. of water containing 3.6 mg. of hydroquinone. When temperature equilibrium had been achieved, the hydroquinone solution was tipped into the elementary body-cytochrome oxidase mixture, and the manometers read at frequent intervals for



TEXT-FIG. 1. The oxidation of cysteine by purified elementary bodies of vaccinia.

oxygen uptake. A control, with cytochrome oxidase and hydroquinone alone, was set up at the same time.

No appreciable oxygen uptake of the elementary body-cytochrome oxidase-hydroquinone mixture, over that in the control flasks, was observed after 2 hours when the experiment was discontinued. When the experiment was repeated, paraphenylenediamine being used as a hydrogen donor, a significant oxygen uptake occurred which lasted over a period of 2 hours.

A much greater and more consistent oxygen uptake, however, was demonstrated when cysteine replaced paraphenylenediamine as a reducing agent. In this instance, the oxygen uptake was as great in the control system, which was composed of elementary bodies and cysteine, without the addition of cytochrome oxidase (Text-fig. 1).

1 mg. of fresh elementary bodies was suspended in 0.15 *M* buffer and placed in the bottom of a Warburg flask. In the side arm was placed 1 cc. of an aqueous solution containing 6 mg. of cysteine. Suitable controls, with elementary bodies and buffer

and cystein and buffer, were set up at the same time. After temperature equilibrium had been achieved, the cystein solution in the side arm was tilted into the respirometer flask; the manometers were read at 10 minute intervals for oxygen uptake. The rate of oxygen uptake from the oxidation of cystein by elementary bodies of vaccinia is shown in Text-fig. 1.

Identical rates of cystein oxidation by elementary bodies and by elementary bodies plus cytochrome oxidase indicated that no participation by cytochrome oxidase in this reaction was likely. With paraphenylenediamine, which is known from redox-potential considerations to be less specific than hydroquinone in the enzymatic reduction of cytochrome *c*, the case is not so clear, since no appreciable oxidation of paraphenylenediamine by elementary bodies of vaccinia without added cytochrome oxidase could be demonstrated. Results obtained by the use of paraphenylenediamine are also rendered ambiguous by the fact that its oxidation is catalyzed by traces of cytochromes *a* and *b* which in most cases contaminate preparations of cytochrome oxidase.

Search for a Metallic Catalyst

The catalytic effect of metals on the oxidation of paraphenylenediamine (8) and cystein (11) is well known. That a metallic component was responsible for the catalysis noted above was further indicated by the effect of potassium cyanide which in a concentration of 0.002 molar was effective in completely blocking the reaction. Aa' dipyriddy, which is known to prevent oxidative catalysis by traces of ferric ion, was wholly without effect, indicating that inorganic iron did not play a part in this reaction. Sodium diethyl-dithiocarbamate, however, was effective in completely preventing the oxidation of cystein by elementary bodies of vaccinia. This substance has long been known to block copper catalysis by irreversible combination with the copper ion, and, because of this reaction, can be made specific for the detection of copper if iron is previously bound by pyrophosphate or dipyriddy (12).

The fact that the oxidation of cystein by elementary bodies of vaccinia was blocked effectively by sodium diethyl-dithiocarbamate made it likely that we were dealing with a copper constituent. It must be remembered that in higher concentration sodium diethyl-dithiocarbamate will block the catalytic effect of certain other metals, such as iron, cobalt, and magnesium. That the catalytic effect was due entirely to a metallic component was indicated by the fact that the ash from elementary bodies of vaccinia gave even a higher rate of cystein oxidation than would have been given by the intact virus.

Metallic ions often contaminate reagents, and our first thought was that in the preparation of elementary bodies of vaccinia copper had been introduced as a contaminant. Careful tests of the buffers and other reagents used in each step in the preparation of virus, however, failed to reveal a source of copper as a contaminant. Stainless steel gauze was substituted for bronze for the

scarification process which precedes the seeding of virus on the skin of rabbits without affecting the concentration of copper in the final product. Moreover, repeated washing of the purified virus resulted in no appreciable change in the concentration of the catalytic substance. Since in no instance could steps in the method of purification of the virus be shown to be responsible for the introduction of the relatively large amount of the catalytic substance observed in the purified virus, it was decided to attempt identification of the compound and find whether it was linked with virus activity.

Spectroscopic Demonstration of Copper in Vaccine Virus.—For proof that the catalytic substance in the purified virus was copper, we turned to spectroscopic studies. Emission spectra procured for us by Dr. G. I. Lavin on several samples of purified virus revealed the lines of copper in each instance (Fig. 1).

The spectra were obtained by placing the dried materials in bored carbon electrodes which were then arced with 110 volts of direct current. The lower electrode, containing the test substance, was made the positive one in each case. The photographs were taken with a medium Hilger quartz spectrograph on 10 inch plates.

As a reference substance, 10 mg. of dried egg albumin, containing 0.05 per cent of added copper as copper sulfate, was likewise arced in a bored electrode. The resulting emission spectrum was compared with respect to position and intensity with that obtained from the purified virus. As a second reference substance 10 mg. of dried material separated from the virus in the last stage of purification (final horizontal sediment) was likewise arced, and the resulting emission spectrum was used for comparison.

When the emission spectrum of purified virus was photographed (Fig. 1 *a*) the only lines to increase in intensity, over the residual traces given by the electrodes, were the copper lines at 3247 and 3274 Å, and those given by phosphorus in the region of 2530 to 2555 Å. The copper lines in the purified virus matched in position and intensity those given by the copper added to the egg albumin (Fig. 1 *b*, *d*).

The spectrum obtained from the horizontal sediment (Fig. 1 *c*) ordinarily discarded in the final stage of virus purification is interesting in that it contains a number of lines not seen in the purified virus. The major line at 3302 Å is thought to be due to zinc. Although this line and others due to metallic trace substances are absent from the final virus product, no diminution in intensity of the copper lines occurred. The absence of iron and other metallic constituents of tissue from the purified virus is highly significant in view of the original source of the virus, from animal skin. This is additional evidence that there is no appreciable quantity of impurities in our virus preparations.

Demonstration of Copper by Chemical Means.—For quantitative determination of the copper constituent, several methods were available. The rate of cystein oxidation in the absence of other metallic substances can in itself be made quantitative. Comparison of the rate of cystein oxidation by elementary

bodies of vaccinia with the rate produced by known increments of copper ion revealed a copper content significantly over 0.03 per cent of the dry weight of the virus. For direct chemical determination we were able to employ successfully the method of Sachs *et al.* (12), which could be performed on as little as 15 mg. of elementary bodies.

15 mg. of elementary bodies, dried to constant weight, were ashed at 600°C. in a vitreosil thimble with the aid of 0.25 cc. of reagent nitric acid. When ashing was complete, as revealed by the complete disappearance of carbon, the ash was extracted with 3 cc. of 6 N hydrochloric acid in 1 cc. amounts by warming the mixture to insure solution. The material was then transferred quantitatively to a 25 cc. volumetric cylinder, and followed by 2 additional rinses of the vitreosil thimble with 1 cc. of distilled water. A blank determination on reagents, including the ashing procedure, was run at the same time. 2 cc. of concentrated ammonia water was added and the solution cooled. 1 cc. of a 0.2 per cent aqueous solution of sodium diethyl-dithiocarbamate was next added and mixed thoroughly with the test solu-

TABLE I
Copper Content of Elementary Bodies of Vaccinia

| Lot | Virus taken for analysis | Copper |
|-----|--------------------------|----------|
| | mg. | per cent |
| 1 | 15 | 0.051 |
| 2 | 15 | 0.056 |
| 3 | 15 | 0.052 |
| 4 | 15 | 0.048 |

tion by rotation of the tube. 10 cc. of isoamyl alcohol were next added, and the mixture shaken thoroughly for 1 minute. The yellow complex of copper and diethyl-dithiocarbamate was extracted quantitatively by the isoamyl alcohol and appeared in the isoamyl alcohol layer which rose slowly to the top and formed a sharp interface with the aqueous layer below. The colored layer was removed by means of a pipette and transferred to a colorimeter cup and compared with a known copper standard treated in an identical manner. All reagents were tested for copper and made up in triple glass-distilled water. No test for copper could be obtained on the reagents alone.

The results of copper determinations made on four different lots of purified elementary bodies are given in Table I.

Nature of Copper Constituent

The fact that the copper constituent of our virus preparations was not appreciably altered by repeated washing of the purified virus indicated that the substance was held more firmly than by mere adsorption of inorganic copper ion.

Ultrafiltration of Elementary Bodies of Vaccinia.—Ultrafiltration, in which the volume of wash water could be kept small, failed to lower significantly the

copper constituent, indicating that it was held firmly to the virus. Copper sulfate, as a source of copper ion, added in amounts 4 to 5 times that present in the virus, was readily removed in two washings.

10 mg. of elementary bodies was suspended in 10 cc. of distilled water and placed in a copper-free cellophane bag made of Visking sausage casing. The upper end was knotted, and the bag placed in an ultrafiltration tube similar to that described by Coolidge (13). The tube was then placed in a centrifuge and spun for 2 hours at 3500 R.P.M. After that time a measurable quantity of ultrafiltrate had appeared in the bottom of the tube below the glass constriction on which the cellophane tube rested. This ultrafiltrate was tested for copper by means of the cystein oxidation technique and resultant oxygen uptake in the Warburg respirometer. No significant trace of copper ion could be detected in the ultrafiltrate, and no diminution in copper concentration was detectable in the elementary bodies from which the ultrafiltrate had been removed. Added copper in amounts twice that already present in the virus, however, was readily removed by such treatment and could be recovered quantitatively in the ultrafiltrate.

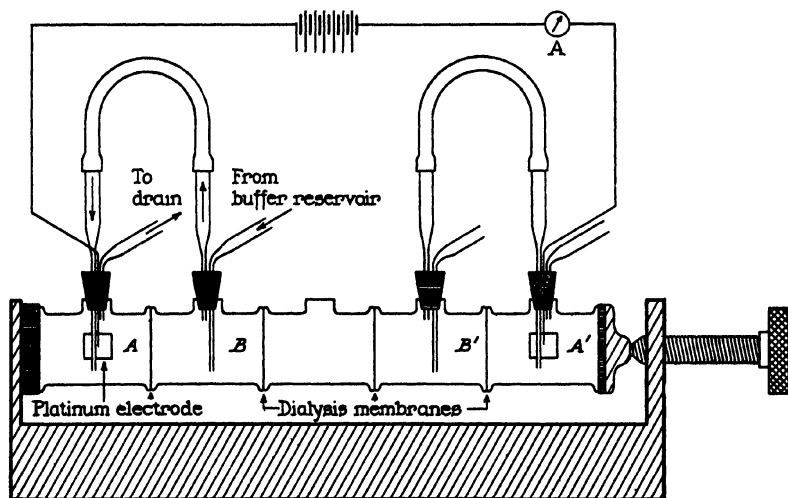
Electrodialysis of Elementary Bodies.—Electrodialysis is one means of insuring removal from proteins of metallic substances which are not held in organic combination. The copper in certain of the purified copper proteins, such as tyrosinase and laccase, is not removed by this procedure. In our experience, the usual methods of electrodialysis render vaccine virus inactive. With ordinary electrodes this can be traced to rapid and uncontrollable pH changes due to electrolysis of buffer. Moreover, since the removal of metallic substances by electrodialysis is also affected greatly by small pH changes, it was necessary to devise some technique whereby pH could be carefully controlled and shifted easily by a variety of buffers. An electrodialysis unit, devised several years ago by Dr. J. H. Bauer and Dr. T. P. Hughes of the International Health Division of The Rockefeller Foundation, was modified according to the accompanying illustration and found quite satisfactory for our purposes.

5 glass cells, with ground contact joints, were separated by cellophane discs which were sealed to the contact joints by rubber cement. A platinum electrode was placed in each of the end cells and buffer of any desired molarity and pH, from a reservoir above, was allowed to run slowly into cells B and B' and over into A and A' by means of glass U-tubes, as shown in Text-fig. 2. From the end cells, the buffer was allowed to drain away at a rate which was controlled by two screw clamps attached to the outlet tubes. The material to be dialyzed was placed in the middle cell, separated from the electrode cells by cells B and B' which were slowly but continuously rinsed with new buffer. pH determinations, done at frequent intervals with the glass electrode, revealed no changes in pH, either in the center or two adjacent cells, over a period of 3 to 4 days of continuous electrodialysis.

10 mg. of freshly prepared elementary bodies of vaccinia were suspended in $M/20$

citrate-NaOH buffer, and submitted to continuous electro dialysis for 36 hours at 20°C., with a potential of 110 volts and a current of 20 milliamperes. Samples of the suspension of virus were removed for copper analyses and infectivity studies at 6 hour intervals.

With $m/20$ citrate-NaOH buffers, over a pH range of 6 to 8.5 no drop in copper concentration was observed. Moreover, no drop in infectivity of the virus over control samples of virus kept at the same temperature and pH was noted. Copper added to egg albumin in one instance and washed suspensions



TEXT-FIG. 2. Modified Hughes-Bauer electro dialysis apparatus in which the pH is controlled by means of circulating dilute buffer.

of *Lactobacillus casei* in another, in amounts equivalent to that found in vaccine virus, was readily and completely removed within 4 to 6 hours over the same pH range. Finally, copper ion added to virus suspensions could likewise be removed by this technique.

Concentration of Copper Constituent with Purification of Virus.—A study of the virus material at various stages of purification has yielded certain information concerning the constituents which make up the purified virus. The virus constituents are necessarily concentrated as the purification proceeds, while the constituents representing contaminants arising from the skin of the rabbit tend to disappear (1). A study of the virus material during the process of purification showed a striking increase in the amount of copper.

A portion of the dermal pulp scraped from the skin of rabbits infected with vaccinia was separated from the virus by differential centrifugation, as described in an

copper is bound with a degree of firmness exhibited by known copper proteins, such as tyrosinase and hemocyanin. It is tempting to assume that a metallic group such as copper, by virtue of its known rôle as an oxidative catalyst, may possibly function in the respiratory activity of the virus. No proof of such a function can be furnished at this time.

SUMMARY

A search by means of spectroscopic and enzymatic techniques has failed to demonstrate either cytochrome or cytochrome oxidase in purified elementary bodies of vaccinia. A constituent of the virus which catalyzes the oxidation of cystein has been found and identified as copper in a concentration amounting to 0.05 per cent of the dry weight of the virus. The copper constituent was not removed by repeated washing, ultrafiltration, dialysis against 0.1 molar potassium cyanide, or by electrodialysis over a pH range which did not inactivate the virus. During the process of purification of the virus a 25-fold increase of the copper constituent was observed. Emission spectra obtained from the dry virus also revealed copper but no significant traces of other metallic substances. No biological rôle can yet be ascribed to the copper component of virus.

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EXPLANATION OF PLATE 6

FIG. 1. Emission spectra of purified elementary bodies of vaccinia (a), of egg albumin to which copper was added to 0.05 per cent concentration (b, d), and of material removed from elementary bodies of vaccinia in final stage of purification (c).

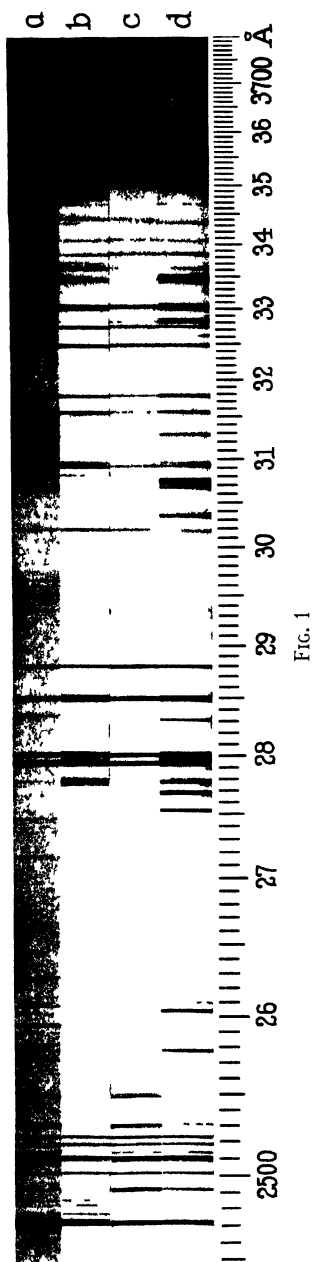


FIG. 1

(Hoagland *et al* Copper constituent in vaccine virus)

THE RELATION OF HIGH AND LOW UREA CLEARANCES TO THE INULIN AND CREATININE CLEARANCES IN CHILDREN WITH THE NEPHROTIC SYNDROME

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During the past 6 years we have observed that, in children with the nephrotic syndrome, the urea clearance is not infrequently increased for periods of 1 or more months to 140 per cent or more of the average clearance of normal children of similar age and size. The phenomenon has occurred with the same frequency in both sexes. In a group of 33 nephrotic children all less than 10 years of age who were admitted to the Hospital, we have seen this type of elevation in 14 (or 42 per cent) of the patients. In 6 of the 14 patients the elevation has persisted for a period of at least 6 months; in 1 of our nephrotic children the urea clearance has been consistently elevated to between 200 and 300 per cent of normal for 6 years. These nephrotic children have been found by Farr (1) to show also a degree of lability of the urea clearance not noted in normal adult man (2) or, in our experience and in that of others, in children or adults (3) with decreased urea clearances. When the dietary protein was reduced from the optimum intake of 3 grams per kilo to 1 gram or less, the urea clearance showed a parallel fall. In contrast to our young children, only 2 out of a group of 54 nephrotic adults and children over 10 years of age observed in this Hospital have shown high clearances; these were aged 11 and 18 years.

The mechanism of this high urea clearance in nephrotic children has not been explained. In the present study we have sought to determine whether the increased urea clearance is accompanied by a similar increase in the inulin clearance, which is believed to equal the volume of the glomerular filtrate (4). We have also determined the ratios of inulin clearance to urea clearance and to creatinine clearance in these patients, and compared them with the same ratios in nephrotic children with diminished urea clearances, and in children who have recovered from acute nephritis.

Patients Studied and Experimental Procedures Followed

The patients studied in our experiments were 3 nephrotic children (R. Q., S. G., R. M.) with high urea clearances, 2 nephrotic children (J. C., S. W.) and 1 nephrotic adult (A. C.) with low urea clearances and, as controls with normal renal function, 2 children who had recovered from acute hemorrhagic Bright's disease. Of the 3

patients in the high clearance group, 1, R. M., had a urea clearance consistently elevated to above 140 per cent of normal; the other 2 patients had urea clearances always above 100 per cent of normal and frequently above 140 per cent. All the nephrotic patients exhibited proteinuria and hyperlipemia and had plasma albumin levels below 2.5 grams per 100 cc. Edema had been present previously in each case but was observed only in S. G. at the time of these experiments. Detailed laboratory and clinical data on 4 of these patients (R. Q., S. G., J. C., S. W.) have been published elsewhere (5, 6). For several months previous to these experiments all of the patients except B. D. and A. C. had been fed a diet which provided 3 grams of protein per kilogram of ideal body weight. The daily intake of sodium chloride was 1 to 1.5 grams.

All tests were performed under fasting conditions; the subjects were kept in bed during the clearance periods. Preceding and during the experiments, from 1 to 2 liters of water were administered orally to maintain an adequate flow of urine. The patients were not catheterized. After 2 or 3 control periods, each of approximately 1 hour's duration, during which specimens of urine and a single blood sample were obtained for the determination of urea and "endogenous" creatinine clearances, creatinine was administered orally. One hour later a single injection of inulin, prepared as a 10 per cent solution in 0.85 per cent sodium chloride solution,¹ was administered intravenously during the course of 15 to 20 minutes. The quantities of creatinine and inulin given varied in the individual experiments as shown in Tables I, II and III. Thirty to 60 minutes following the injection of inulin, urine collections were resumed for the determination of simultaneous inulin, "exogenous" creatinine, and urea clearances. The duration of these latter periods of urine collection varied usually from 30 to 60 minutes and was determined by the patients' desire to void. Venous blood samples were obtained at the beginning and end of each period.

Casein hydrolysate,² prepared as a 10 per cent solution (6, 7) was given intravenously on at least one occasion to each of the low-clearance patients, and to S. G. in the high-clearance group. The amino acid mixture was given after 1 or 2 periods of simultaneous determination of inulin, urea, and creatinine clearances, and all clearances were again determined during 1 or 2 subsequent periods. The quantities of casein hydrolysate given to each patient are shown in Tables I and II.

Analytical Methods

Urea-plus-ammonia nitrogen in whole blood and urine was determined in some experiments by the hypobromite gasometric method of Van Slyke and Kugel (8); the experiments in which this method was used are indicated by an asterisk in Tables I and II. In the remainder plasma and urine urea nitrogen were determined by the gasometric urease method of Van Slyke (9).

Creatinine was determined with the Summerson (10) photoelectric colorimeter by the method of Folin and Wu as modified by Miller and Winkler (11). The plasma values of the 2 control patients were corrected for non-creatinine chromogen by the

¹ The 10 per cent solution of inulin in saline was purchased from the U. S. Standard Products Co., Woodworth, Wisconsin.

² Furnished through the generosity of Mead Johnson and Co., Evansville, Indiana.

specific enzymatic method of Miller and Dubos (12). In the high-clearance group the "endogenous" plasma chromogen levels were so low as to make accurate determination of non-creatinine chromogen impossible; indeed, in the case of R. M. there was no Jaffe reaction demonstrable in the plasma filtrate. In the single low-clearance patient (J. C.) in which it was determined, the non-creatinine chromogen was less than 10 per cent of the total chromogenic substance of the plasma; no corrections have been applied to the plasma values of the low-clearance group.

Plasma and urinary inulin were determined by the technique described by Alving, Rubin and Miller (13), which was modified slightly according to suggestions made by Dr. A. S. Alving in personal communications; the Summerson photoelectric colorimeter was employed.

CALCULATIONS

The urea, creatinine, and inulin clearances were calculated as the number of cc. of plasma cleared per minute per 1.73 square meters of surface area. The formula³ of Møller, McIntosh and Van Slyke (14) was used. We have termed "endogenous" the creatinine clearances which were measured without administration of creatinine, and "exogenous" those which were determined after the blood creatinine content was increased by the feeding of creatinine. The values for the plasma concentrations of inulin and creatinine, in experiments where these substances were administered, were estimated for the midpoint of each period by interpolation on a graph, plasma con-

³ The general clearance formula, introduced by Møller, McIntosh, and Van Slyke (14), is:

$$\text{Clearance} = \frac{U V}{P}.$$

U and P are, respectively, the concentrations in urine and plasma of the excreted substance—urea, creatinine, or inulin, etc.—and V is the urine flow expressed as cc. per minute per 1.73 square meters of body surface. The use of surface area in this calculation, as in the calculation of McIntosh, Møller and Van Slyke (15), makes the clearance formula give the same normal values for infants and children as for adults (15, 16). The surface area used in the calculation is estimated from the height and age of the child, as described by McIntosh *et al.* (15).

When the urine volume is above 2 cc. per minute per 1.73 square meters of body area, the urea clearance in man is independent of volume change; hence, urea clearances with V above 2 cc. have been termed "maximum" clearances (14). When V is between 0.5 and 2 cc., the urea clearance has been found to vary as the square root of the urine volume (14). Hence, for volumes within this range, the maximum urea

clearance is calculated as $\frac{U}{P} \times V \times \sqrt{\frac{2}{V}}$ or $\frac{U}{P} \sqrt{2V}$ in this paper. The clear-

ances of inulin and creatinine are calculated simply as $\frac{U V}{P}$ for all urine volumes, since it was noted that a fall of V to the range between 2 and 0.5 cc. did not decrease the clearance of inulin or creatinine.

TABLE I

Nephrotic Patients with High Urea Clearances. Comparison of Inulin, Creatinine, and Urea Clearances

| Subject | Period | Duration | Urine flow V†† | Plasma levels | | | Urine levels | | | Clearances | | | | Clearance ratios | |
|-----------------------------------------------------------------|---------|----------|----------------|------------------|------------------|------------------|------------------|------------------|------------------|-----------------------|-----------------------|-----------------------|-----------------------|------------------------------|--------------|
| | | | | Inulin | Creatinine | Urea N | Inulin | Creatinine | Urea N | Inulin | Endogenous creatinine | Exogenous creatinine | Urea | Exogenous creatinine: Inulin | Urea: Inulin |
| | | min-utes | cc. per minute | mgm. per 100 cc. | mgm. per 100 cc. | mgm. per 100 cc. | mgm. per 100 cc. | mgm. per 100 cc. | mgm. per 100 cc. | cc. plasma per minute | cc. plasma per minute | cc. plasma per minute | cc. plasma per minute | | |
| R. Q. ♀ 8 years (V factor†† = 1.74) January 25, 1940 | 1 | 60 | 3.61 | | | 7.5* | | | 230* | | | | 111* | | |
| | 2§ | 55 | 4.30 | | | 6.2* | | | 177* | | | | 122* | | |
| | 3 | 60 | 6.68 | 11.6 | | 6.2* | 330 | | 105* | 190 | | | 113* | 0.59* | |
| | 4 | 60 | 3.55 | 4.4 | | 6.4* | 280 | | 192* | 226 | | | 106* | 0.47* | |
| | Average | | | | | | | | | 208 | | | 113 | | |
| Idem March 11, 1940 | 1 | 70 | 6.72 | | 0.33 | 7.6 | | 9.2 | 146 | | 184 | | 129 | | |
| | 2 | 57 | 3.56 | | | | | 15.6 | 194 | | 166 | | 91 | | |
| | 3 | 58 | 4.14 | | | | | 14.0 | 208 | | 173 | | 113 | | |
| | 4¶ | 59 | 2.32 | | 3.45 | 7.5 | | 117.4 | 282 | | | | 87 | | |
| | 5 | 75 | 6.10 | | 7.38 | 7.0 | | 340.0 | 105 | | | 281 | 92 | | |
| | 6 | 33 | 1.03 | 21.0 | 5.45 | 7.0 | 4300 | 2030.0 | 418 | 212 | | 386 | 86 | 1.82 | 0.41 |
| | 7 | 32 | 2.40 | 14.6 | 4.55 | 6.5 | 1200 | 586.0 | 256 | 197 | | 309 | 95 | 1.57 | 0.48 |
| | 8 | 41 | 3.84 | 10.4 | 3.78 | 6.5 | 450 | 220.0 | 128 | 166 | | 223 | 75 | 1.34 | 0.45 |
| | 9 | 34 | 7.64 | 7.2 | 3.00 | 7.2 | 185 | 114.0 | 103 | 196 | | 290 | 110 | 1.48 | 0.56 |
| | Average | | | | | | | | | 193 | 174 | 297 | 98 | | |
| S. G. ♂ 10 years (V factor†† = 1.75) February 26, 1940 | 1 | 114 | 2.01 | | 0.28 | 9.1 | | 24.7 | 418 | | 177 | | 92 | | |
| | 2 | 61 | 4.70 | | | | | 11.7 | 202 | | 197 | | 104 | | |
| | 3¶ | 60 | 3.04 | | 2.30 | | | 295.0 | 262 | | | 390† | 88 | | |
| | 4 | 60 | 6.25 | | 3.67 | 8.9 | | 216.0 | 123 | | | 368 | 86 | | |
| | 5 | 60 | 1.81 | 18.1 | 2.26 | 9.1 | 1520 | 420.0 | 346 | 152 | | 336 | 72 | 2.21 | 0.47 |
| | 6 | 60 | 1.20 | 8.7 | 1.32 | 9.4 | 1380 | 440.0 | 468 | 190 | | 370 | 77 | 1.95 | 0.41 |
| | 7†† | 62 | 0.62 | 5.6 | 0.95 | 12.8 | 1160 | 403.0 | 316 | 129†† | | 263†† | 28†† | 2.04 | 0.22 |
| | 8 | 58 | 1.69 | 3.2 | 0.75 | 14.4 | 620 | 123.0 | 478 | 327 | | 276 | 61 | 0.84 | 0.19 |
| | Average | | | | | | | | | 171 | 187 | 366 | 86 | | |

* In these experiments, whole blood and urine were analyzed for urea-plus-ammonia nitrogen by the gasometric hypobromite method. The use of the hypobromite method in analyzing whole blood and urine has been demonstrated in our high-clearance patients to furnish whole blood clearance results not deviating by more than 10 per cent from simultaneous plasma clearance determinations in which plasma and urine were analyzed by the urease method. Hence, all clearance values have been tabulated as "plasma" clearances, and used as such in calculating ratios.

† Endogenous plasma creatinine too low to measure.

‡ Plasma creatinine level rising during this period.

TABLE I—*Concluded*

| Subject | Period | Duration | Urine flow $V^{\dagger\dagger}$ | Plasma levels | | | Urine levels | | | Clearances | | | | Clearance ratios | |
|-----------------------------------------------------------------------------------|---------|----------|---------------------------------|------------------|------------------|------------------|------------------|------------------|------------------|-------------------------|-------------------------|-------------------------|-------------------------|------------------------------|--------------|
| | | | | Inulin | Creatinine | Urea N | Inulin | Creatinine | Urea N | Inulin | Endogenous creatinine | Exogenous creatinine | Urea | Exogenous creatinine: Inulin | Urea: Inulin |
| | | min-utes | cc. per min-ute | mgm. per 100 cc. | mgm. per 100 cc. | mgm. per 100 cc. | mgm. per 100 cc. | mgm. per 100 cc. | mgm. per 100 cc. | cc. plas-ma per min-ute | cc. plas-ma per min-ute | cc. plas-ma per min-ute | cc. plas-ma per min-ute | | |
| R. M. ♂ 3 years (V factor $^{\dagger\dagger} = 2.75$) April 16, 1940 | 1 | 55 | 2.99 | | † | 7.1 | | | | 323 | | † | | 136 | |
| | 2 | 60 | 11.10 | | | | | | | 76 | | | | 119 | |
| | 3 | 58 | 6.55 | | | | | | | 116 | | | | 103 | |
| | 4** | 52 | 3.59 | | 3.60 | | | 334.0 | | 207 | | 333‡ | | 105 | |
| | 5 | 50 | 7.05 | | 11.96 | 6.7 | | 634.0 | | 101 | | 373 | | 105 | |
| | 6 | 35 | 10.80 | 51.5 | 11.12 | 6.7 | 1030 | 358.0 | 72 | 216 | | 349 | 115 | 1.62 | 0.53 |
| | 7 | 30 | 6.96 | 20.0 | 9.40 | 6.6 | 760 | 400.0 | 95 | 265 | | 296 | 100 | 1.12 | 0.38 |
| | 8 | 30 | 1.46 | 14.3 | 7.05 | 6.6 | 2300 | 1672.0 | 467 | 236 | | 348 | 121 | 1.47 | 0.51 |
| | 9 | 45 | 7.53 | 9.8 | 5.26 | 6.7 | 270 | 195.0 | 104 | 207 | | 279 | 116 | 1.35 | 0.56 |
| | 10 | 30 | 5.68 | 5.0 | 3.70 | 7.0 | 200 | 170.0 | 104 | 237 | | 261 | 85 | 1.10 | 0.36 |
| | Average | | | | | | | | | | 233 | | 320 | 111 | |

§ Inulin 5 grams given intravenously during this period.

|| Inulin 10 grams given intravenously during this period.

¶ Creatinine 4 grams given by mouth during this period.

** Creatinine 5 grams given by mouth during this period.

†† Casein hydrolysate 20 grams given intravenously during this period, with subsequent chill and rise of temperature to 103.4°. Clearance values in Periods 7 and 8 not used in calculating averages.

‡‡ To obtain " V ," which is the urine flow per minute per 1.73 square meters of body surface, the observed urine flow has been multiplied by the " V factor," which is the ratio of 1.73 to the subject's surface area in square meters, determined from his age and height (15).

centration being plotted arithmetically against time. In each instance, the reported values for the inulin clearances were estimated from data obtained while the plasma level of inulin was falling; calculations of exogenous creatinine clearances were likewise made from data obtained while the plasma creatinine concentration was falling, except where it is specifically stated otherwise in the tables. Since blood urea changed but little during the experiment, the plasma urea value directly obtained at the end of each period was used in calculating the urea clearance for that period.

RESULTS

Comparison of Urea, Inulin and Creatinine Clearances

The results are given in detail, with respect to each patient of the 3 groups, in Tables I, II and III. In general, *all clearances were affected similarly in each*

TABLE II

Nephrotic Patients with Low Urea Clearances. Comparison of Inulin, Creatinine, and Urea Clearances

| Subject | Period | Duration | Urine flow V§§ | Plasma levels | | | Urine levels | | | Clearances | | | | Clearance ratios | |
|-----------------------------------------------------------------|---------|----------|-----------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------------|------------------------|------------------------|------------------------|-------------------------------|------------------------------|
| | | | | Inulin | Creatinine | Urea N | Inulin | Creatinine | Urea N | Inulin | Endogenous creatinine | Exogenous creatinine | Urea | Endogenous creatinine: Inulin | Exogenous creatinine: Inulin |
| | | min-utes | cc. per min-ute | mgm. per 100 cc. | mgm. per 100 cc. | mgm. per 100 cc. | mgm. per 100 cc. | mgm. per 100 cc. | mgm. per 100 cc. | cc. plasma per min-ute | cc. plasma per min-ute | cc. plasma per min-ute | cc. plasma per min-ute | | |
| A. C. ♀ 21 years (V factor§§ = 1.03) February 5, 1940 | 1 | 84 | 2.06 | | 4.0 | 33.7* | | 34.2 | 203* | | 17.6 | | 12.3* | | |
| | 2§ | 60 | 2.65 | | | 32.7* | | 29.1 | 178* | | 19.7 | | 14.4* | | |
| | 3 | 60 | 3.57 | 30.5 | | 34.4* | 125 | 22.8 | 135* | 14.7 | 21.0 | | 14.0* | 1.43 | 0.95* |
| | 4 | 60 | 3.04 | 25.5 | | 33.6* | 120 | 25.1 | 152* | 14.3 | 19.2 | | 13.7* | 1.34 | 0.96* |
| | Average | | | | | | | | | 14.5 | 19.4 | | 13.6* | | |
| Idem February 10, 1940 | 1 | 62 | 2.28 | | 4.1 | 32.9* | | 33.0 | 188* | | 18.5 | | 13.2* | | |
| | 2 | 60 | 3.69 | | | | | 24.1 | 114* | | 19.7 | | 12.9* | | |
| | 3 | 57 | 3.92 | | | | | 21.2 | 111* | | 20.1 | | 13.4* | | |
| | 4 | 60 | 1.88 | | | | | 34.3 | 203* | | 15.7 | | 12.0* | | |
| | 5 | 62 | 3.30 | | 8.1 | | | 32.3 | 131* | | | | 13.2* | | |
| | 6 | 64 | 3.24 | 37.7 | 14.2 | 31.8* | 155 | 65.5 | 127* | 13.5 | | 14.9† | 13.0* | 1.10 | 0.96* |
| | 7 | 59 | 2.65 | 31.2 | 16.2 | 31.5* | 165 | 108.4 | 152* | 14.1 | | 17.7 | 12.9* | 1.26 | 0.91* |
| | 8†† | 58 | 3.84 | 28.2 | 15.8 | 32.5* | 113 | 73.0 | 110* | 15.5 | | 17.7 | 13.1* | 1.14 | 0.85* |
| | 9 | 62 | 2.52 | 27.0 | 15.4 | 32.6* | 128 | 94.9 | 155* | 12.0 | | 15.5 | 12.1* | 1.29 | 1.01* |
| | Average | | | | | | | | | 13.8 | 18.5 | 16.5 | 12.9* | | |
| S. W. ♀, 4 years (V factor§§ = 2.95) February 13, 1940 | 1 | 57 | 2.28 | | 2.7 | 44.6* | | 15.2 | 223* | | 12.8 | | 11.4* | | |
| | 2 | 55 | 4.07 | | | | | 9.7 | 143* | | 14.7 | | 13.0* | | |
| | 2 | 61 | 2.13 | | | | | 15.5 | 224* | | 12.3 | | 10.7* | | |
| | 4‡ | 62 | 1.90 | | 4.3 | | | 35.0 | 238* | | | | 10.4* | | |
| | 5 | 68 | 2.17 | 44.1 | 10.4 | 43.5* | 282 | 82.7 | 193* | 13.9 | | 17.2† | 9.6* | 1.24 | 0.69* |
| | 6 | 54 | 2.41 | 39.8 | 13.5 | 42.6* | 205 | 88.4 | 174* | 12.4 | | 15.7 | 9.9* | 1.27 | 0.80* |
| | 7†† | 62 | 2.09 | 35.8 | 12.8 | 46.0* | 205 | 94.5 | 212* | 12.0 | | 15.4 | 9.6* | 1.28 | 0.80* |
| | 8 | 53 | 2.50 | 32.0 | 12.0 | 44.4* | 140 | 76.8 | 193* | 10.9 | | 16.3 | 10.5* | 1.50 | 0.96* |
| | Average | | | | | | | | | 12.3 | 13.3 | 16.1 | 10.6* | | |

* See asterisked footnote Table I.

† Plasma creatinine level rising during this period.

‡ Inulin 3.5 grams given intravenously and creatinine 1 gram given by mouth during this period.

§ Inulin 5 grams given intravenously during this period.

|| Inulin 5 grams given intravenously and creatinine 4 grams given by mouth during this period.

¶ Creatinine 2 grams given by mouth during this period.

** Creatinine 5 grams given by mouth during this period.

†† Casein hydrolysate 5 grams given intravenously during this period.

‡‡ Casein hydrolysate 10 grams given intravenously during this period.

§§ See footnote ‡‡, Table I.

TABLE II—*Concluded*

| Subject | Period | Duration | Urine flow V% cc. per min-ute | Plasma levels | | | Urine levels | | | Clearances | | | | Clearance ratios | |
|-----------------------------------------------------------------------------|-----------------|----------|----------------------------------|----------------------------|--------------------------------|----------------------------|----------------------------|--------------------------------|----------------------------|---------------------------------------|-----------------------------------------------------------|----------------------------------------------------------|-------------------------------------|------------------------------------|-----------------------------------|
| | | | | Inulin mgm. per 100 cc. | Creatinine mgm. per 100 cc. | Urea N mgm. per 100 cc. | Inulin mgm. per 100 cc. | Creatinine mgm. per 100 cc. | Urea N mgm. per 100 cc. | Inulin cc. plas- ma per min-ute | Endogenous creati- nine cc. plas- ma per min-ute | Exogenous creati- nine cc. plas- ma per min-ute | Urea cc. plas- ma per min-ute | Endogenous creati- nine: Inulin | Exogenous creati- nine: Inulin |
| J. C. ♂ 7 years (V factor $\frac{1}{100}$ = 2.26) January 29, 1940 | 1 | 104 | 2.77 | | 2.0 | 38.1* | | | 19.0 | 227* | 25.8 | | 16.5* | | |
| | 2 | 47 | 4.23 | | 1.9 | | | | 12.5 | 136* | 27.1 | | 15.2* | | |
| | 3 $\frac{1}{2}$ | 74 | 4.70 | | | 37.0* | | | 11.2 | 130* | 27.1 | | 16.5* | | |
| | 4 | 60 | 4.74 | 63.3 | 2.0 | 37.2* | 260 | 11.1 | 121* | 19.6 | 26.1 | | 15.4* | 1.33 | 0.79* |
| | 5 | 61 | 3.63 | 52.7 | | 36.9* | 240 | 12.9 | 149* | 16.5 | 23.3 | | 14.5* | 1.41 | 0.88* |
| | Average | | | | | | | | | | 18.0 | 25.9 | 15.6* | | |
| Idem February 1, 1940 | 1 | 101 | 3.14 | | 2.0 | | | | 15.4 | | 24.2 | | | | |
| | 2 | 80 | 4.74 | | | 39.6* | | | 8.9 | 120* | 21.2 | | 14.4* | | |
| | 3** | 54 | 5.35 | | 9.9 | | | | 52.4 | 118* | | | 15.9* | | |
| | 5 $\frac{1}{2}$ | 61 | 4.70 | | 18.1 | 38.8* | | | 107.2 | 126* | 28.2† | | 15.2* | | |
| | 5 | 60 | 5.63 | 60.5 | 18.0 | 37.1* | 200 | 96.0 | 115* | 18.5 | 30.0 | | 17.5* | 1.62 | 0.95* |
| | 6†† | 63 | 4.03 | 50.0 | 17.2 | 41.0* | 220 | 116.0 | 162* | 17.7 | 27.1 | | 16.0* | 1.53 | 0.90* |
| | 7 | 57 | 5.05 | 44.4 | 16.7 | 41.9* | 140 | 88.7 | 135* | 16.0 | 26.9 | | 16.3* | 1.68 | 1.02* |
| | Average | | | | | | | | | | 17.4 | 22.7 | 15.9* | | |
| Idem March 7, 1940 | 1 | 61 | 9.05 | | 4.6 | 100.0 | | | 18.1 | 260 | 35.9 | | 23.5 | | |
| | 2 | 60 | 6.68 | | | | | | 17.5 | 247 | 25.5 | | 16.5 | | |
| | 3 | 76 | 0.38 | | | | | | 12.1 | 152 | | | 1.3 | | |
| | 4 $\frac{1}{2}$ | 54 | 6.84 | | 10.0 | | | | 21.5 | 203 | | | 13.9 | | |
| | 5 $\frac{1}{2}$ | 66 | 3.62 | | 17.1 | 98.6 | | | 51.3 | 182 | | 10.9† | 6.5 | | |
| | 6 | 59 | 3.58 | 82.0 | 17.6 | | 205 | 50.6 | 176 | 9.0 | 10.4 | | 6.4 | 1.16 | 0.71 |
| | 7 | 70 | 3.60 | 74.0 | 16.6 | 92.5 | 170 | 46.9 | 178 | 8.3 | 10.2 | | 6.9 | 1.23 | 0.83 |
| | 8†† | 140 | 2.99 | 60.0 | 16.0 | 96.4 | 195 | 55.9 | 222 | 9.7 | 10.5 | | 6.9 | 1.08 | 0.71 |
| | Average | | | | | | | | | | — | — | — | | |

group of patients. Those patients with a high urea clearance had elevated creatinine and inulin clearances. In the control group all three clearances were within the usual ranges of normal values (4, 15, 16, 17). In the low-clearance group all three clearances were depressed. The consistency of the results in each group is best demonstrated by the ratio of urea clearance to inulin clearance, which is also shown in Tables I, II, and III. The use of the ratio for comparative purposes compensates in part for divergencies in calculated results due to incomplete voiding of urine.

J. C. (Table II) failed to excrete ingested water during the experiment of March 7.

An abrupt drop in clearances occurred during the experiment. Soon after the close of Period 8 the patient manifested a generalized convulsion which we believe was a result of water retention.

TABLE III

Control Subjects (Recovered Group). Comparison of Inulin, Creatinine, and Urea Clearances

| Subject | Period | Duration | Urine flow $V\frac{1}{2}$ | Plasma levels | | | Urine levels | | | Clearances | | | | Clearance ratios | |
|------------------------------------------------------------|---------|----------|---------------------------|------------------|------------------|------------------|------------------|------------------|------------------|-----------------------|-----------------------|-----------------------|-----------------------|------------------------------|--------------|
| | | | | Inulin | Creatinine | Urea N | Inulin | Creatinine | Urea N | Inulin | Endogenous creatinine | Exogenous creatinine | Urea | Exogenous creatinine: Inulin | Urea: Inulin |
| | | min-utes | cc. per minute | mgm. per 100 cc. | mgm. per 100 cc. | mgm. per 100 cc. | mgm. per 100 cc. | mgm. per 100 cc. | mgm. per 100 cc. | cc. plasma per minute | cc. plasma per minute | cc. plasma per minute | cc. plasma per minute | | |
| H. G. ♀ 5 years (V factor§ = 2.10) April 22, 1940 | 1 | 60 | 1.47 | | 0.48 | 14.6 | | 49.6 | 1170 | | 151 | | 138 | | |
| | 2 | 60 | 9.11 | | | | | 6.0 | 121 | | 112 | | 75 | | |
| | 3 | 58 | 2.97 | | | | | 9.4 | 150 | | 58 | | 30 | | |
| | 4† | 60 | 3.25 | | 0.70 | | | 18.1 | 322 | | | | 72 | | |
| | 5* | 50 | 5.46 | | 5.98 | 13.4 | | 162.0 | 152 | | | | 62 | | |
| | 6 | 32 | 1.71 | 48.6 | 13.25 | 13.2 | 4160 | 1300.0 | 740 | 146 | | 167 | 103 | 1.14 | 0.71 |
| | 7 | 30 | 3.42 | 28.1 | 11.12 | 12.5 | 1200 | 570.0 | 278 | 147 | | 176 | 76 | 1.20 | 0.52 |
| | 8 | 31 | 13.50 | 18.8 | 8.92 | 12.4 | 157 | 122.0 | 78 | 113 | | 185 | 78 | 1.64 | 0.69 |
| | 9 | 30 | 15.00 | 13.5 | 7.20 | 12.7 | 84 | 88.8 | 66 | 93 | | 185 | 79 | 1.99 | 0.85 |
| | 10 | 30 | 5.04 | 9.5 | 6.14 | 12.5 | 165 | 165.0 | 144 | 87 | | 135 | 68 | 1.55 | 0.78 |
| | Average | | | | | | | | | | 117 | 107 | 170 | 78 | |
| B. D. ♀ 6 years (V factor§ = 2.12) June 21, 1940 | 1 | 60 | 0.92 | | 0.46 | 13.0 | | 58.2 | 658 | | 116 | | 69 | | |
| | 2 | 51 | 4.63 | | | | | 11.7 | 328 | | 117 | | 117 | | |
| | 3† | 123 | 0.67 | | | | | | 451 | | | | 40 | | |
| | 4* | 63 | 7.42 | | 8.21 | 11.8 | | 197.0 | 148 | | | | 93 | | |
| | 5 | 30 | 2.26 | 57.0 | 10.16 | 11.5 | 3870 | 626.0 | 339 | 154 | | | 67 | | 0.43 |
| | 6 | 30 | 2.48 | 28.0 | 10.34 | 11.2 | 1725 | 737.0 | 324 | 152 | | 176 | 72 | 1.16 | 0.47 |
| | 7 | 31 | 4.45 | 17.5 | 9.40 | 10.9 | 590 | 382.0 | 171 | 150 | | 180 | 70 | 1.20 | 0.47 |
| | 8 | 29 | 4.09 | 12.5 | 8.38 | 9.9 | 450 | 387.0 | 186 | 147 | | 189 | 77 | 1.29 | 0.52 |
| | Average | | | | | | | | | | 151 | 116 | 182 | 76 | |

* Inulin 10 grams given intravenously during this period.

† Creatinine 4 grams given by mouth during this period.

‡ Creatinine 5 grams given by mouth during this period.

§ See footnote ‡, Table I.

Administration of Casein Hydrolysate

The intravenous administration of casein hydrolysate to low-clearance patients was not followed by a rise in the clearance values. When it was given to S. G. he developed a severe chill and hyperpyrexia, with the diverse effects

on the clearances noted in Table I. He had tolerated similar injections without reactions during the preceding 6 months.

DISCUSSION

High Clearance and Glomerular Filtration

Our patients with high urea clearances showed similarly high inulin clearances. Therefore, if we assume with Chasis and Smith (18) that the inulin clearance is an accurate index of glomerular filtration, we may conclude that in our patients the rate of formation of glomerular filtrate was abnormally rapid.

The question remains, whether the doubling of the rate of glomerular filtration was due (1) to doubling of the renal blood flow, with a normal filtration fraction of about 20 per cent (4, 19); (2) to a doubling of the filtered fraction of the plasma water, with a concomitant rise in the "extraction percentage" (19) of inulin and urea; or (3) to a combination of both mechanisms. One might expect that the hypoproteinemia of nephrosis, with resultant decrease in plasma oncotic pressure, would induce glomerular filtration of an increased fraction of the plasma water. We have, however, been unable to find any consistent correlation between low plasma albumin and high urea clearance, since we have observed the high clearance to persist long after normal plasma protein concentration has been regained. It appears, therefore, that some cause more dominant than hypoproteinemia is chiefly responsible.⁴

Tubular Excretion of Creatinine

We have estimated the relative output of creatinine by glomerular filtration and tubular excretion on the assumption that the inulin clearance measured glomerular filtration. From the data of each experiment the rate of total creatinine excretion and the rate of filtration of creatinine (inulin clearance \times plasma creatinine concentration) were calculated as mgm. per minute per 1.73 square meters of body surface and plotted for each period against the plasma creatinine concentration for that period. The periods both with and without creatinine feeding were included. With uniformity the curves obtained approximated straight lines, both for observed total creatinine excretion and for estimated glomerular filtration, at plasma levels up to 10 mgm. per 100 cc.

⁴ There is one type of control that our data lack, *viz.*, the estimation of urea clearances on entirely normal children placed on the same régime of diet (salt poor, 3 grams of protein per kilo) and activity as our patients. The possibility that under these conditions normal children might show higher than ordinary clearances is not excluded by our data, nor by any that we have found in the literature. The absence of such high values in normal children on unrestricted diets (16) makes their occurrence seem improbable, but one cannot say that it is absolutely excluded.

For numerical comparisons of the different subjects, excretions have been calculated for a constant plasma creatinine concentration of 10 mgm. per 100 cc., or 0.1 mgm. per cc. The calculations have been made as follows:

(a) Total mgm. creatinine excreted per minute = (cc. plasma cleared of creatinine per minute) \times 0.1

(b) Mgm. creatinine filtered by glomeruli per minute = (cc. plasma cleared of inulin per minute) \times 0.1

(c) Mgm. creatinine excreted by tubules per minute = $a - b$.

The values for plasma creatinine clearance used in formula a and inulin clearance used in formula b are the means of the determinations in each subject.

TABLE IV

Rate of Excretion of Creatinine by All Subjects Estimated for a Plasma Creatinine Concentration of 10 Mgm. Per 100 Cc., Corrected to a Body Surface Area of 1.73 Square Meters

| Patient | Total | Filtered | Secreted |
|-----------------------------|------------------------|------------------------|------------------------|
| | <i>mgm. per minute</i> | <i>mgm. per minute</i> | <i>mgm. per minute</i> |
| <i>Recovered Group</i> | | | |
| H. G. | 17.0 | 11.7 | 5.3 |
| B. D. | 18.2 | 15.1 | 3.1 |
| Average | 17.6 | 13.4 | 4.2 |
| <i>High-clearance group</i> | | | |
| R. Q. March 11, 1940 | 29.7 | 19.3 | 10.4 |
| S. G. | 36.6 | 17.1 | 19.5 |
| R. M. | 32.0 | 23.3 | 8.7 |
| Average | 32.8 | 19.9 | 12.9 |
| <i>Low-clearance group</i> | | | |
| J. C. February 1, 1940 | 2.80 | 1.74 | 1.06 |
| March 7, 1940 | 1.05* | 0.90* | 0.15* |
| S. W. | 1.61 | 1.23 | 0.38 |
| A. C. | 1.65 | 1.38 | 0.27 |
| Average | 2.02 | 1.45 | 0.57 |

* Not included in average—see text.

The results of the calculations are given in Table IV. They indicate that the estimated tubular excretion of creatinine in the 3 groups parallels glomerular filtration, the mean tubular excretion of the high-clearance group being 12.9 mgm. per minute and that of the low-clearance group 0.6 mgm., compared with 4.2 mgm. for the controls.

CONCLUSIONS

The persistent, abnormally high urea clearance, as great as 150 to 200 per cent of normal average, observed in certain children with the nephrotic syndrome, is a manifestation of generally increased renal excretory activity, since

the inulin and creatinine clearances are also elevated above normal to approximately the same degree.

Insofar as tubular activity can be estimated from the ratio of exogenous creatinine clearance to inulin clearance, it appears that this activity is as much accelerated as is glomerular filtration.

Intravenous injection of amino acids did not increase the clearances in patients with diminished renal function.

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THE EFFECT OF HEAT ON CRYSTALS OF SERUM ALBUMIN; PRODUCTION OF CRYSTALS OF DENATURED PROTEIN

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A study of the effects of heat on crystals of serum albumin has led to some curious and unexpected results. That heat denatures proteins is well known, and many investigations have been made on the heat denaturation of protein solutions. For a study of heat denaturation of proteins *in the crystalline state* horse serum albumin is a suitable protein because crystals of this protein do not tend to dissolve when the temperature rises, as crystals of egg albumin, for example, do. On the contrary, under certain conditions, warming a solution of horse serum albumin increases the rate of crystallization. If 0.5 ml of serum albumin solution (containing 50 mg of protein, previously crystallized, but not fractionated and subsequently dialyzed free of salt and then filtered) is added to 12 ml of a sodium sulfate-acetate solution (made by mixing 10 ml of 23 per cent sodium sulfate with 1 ml of 2 M sodium acetate and 1 ml of 2 M acetic acid), crystallization begins in less than 5 minutes if the solution is warmed to 45° C., and within 15 minutes there appears a plentiful crop of large well-formed, needle-shaped crystals. At 25° crystallization proceeds much more slowly. These crystals dissolve at once if 12 ml of water are added. Heating the original solution of serum albumin in sodium sulfate-acetate to 60°, instead of 45°, causes a large flocculent precipitate of protein to appear almost at once. This precipitate is amorphous and, if heating at 60° is continued for 15 minutes, practically no protein dissolves when the suspension is subsequently cooled to room temperature and 12 ml of water are added. At 60° the amorphous protein precipitate obtained is obviously denatured.

At this point it seemed of interest to inquire what would happen to crystals of serum albumin if they were heated to 60° and still higher temperatures. For this purpose crystallization at 45° was allowed to proceed for several hours. The crystals were separated by centrifuging from the small amount of albumin still remaining in solution, and to the crystals was added the original volume of sodium sulfate-acetate mixture at 45°. In this medium the protein crystals were heated at various temperatures from 60° to 100° and finally in an autoclave at 115°, at each temperature for 15 minutes. At no temperature were the crystals destroyed. Even after heating at 115° the crystals seemed as perfectly formed as before heating.

The solubility of the heated albumin crystals was tested at room temperatures by adding to each heated preparation 2 volumes of water. The crystals

heated at 60° dissolved in the course of five or ten minutes. Since heating a *solution* of serum albumin under the same conditions renders the albumin insoluble, it is clear that the protein in the crystal is not as easily denatured as is dissolved protein. If protein denaturation is an unfolding process, as there is good reason to believe,¹ then the increased stability of the protein in a crystal may be explained by supposing that the tendency of a molecule to unfold as the temperature is raised is opposed by the bolstering effect of neighboring molecules in the crystal.

Crystals of serum albumin heated at temperatures higher than 60° did not dissolve completely. A small percentage of those heated at 70° dissolved, but practically none of the crystals heated between 80° and 115° dissolved even after standing, with occasional stirring, for three days. These albumin crystals were not destroyed by being placed for several days in 1 N HCl or 95 per cent alcohol. They dissolved at once, however, in a saturated urea solution. The insoluble serum albumin crystals are as insoluble as a heat-denatured, amorphous coagulum of serum albumin. One hesitates, however, to compare the heated crystals with a heat denatured protein because, despite many efforts, no denatured protein has been crystallized. And yet it can be shown that the heated, insoluble crystals are indeed crystals of denatured protein. The crystals, washed free of sodium sulfate by repeated centrifugation, readily dissolve in a pH 9.2 borate buffer. Crystals prepared from 0.3 ml of the serum albumin preparation mentioned above can be dissolved in 0.4 ml of a 0.1 M pH 9.2 borate buffer. If to this solution at 45° are added 5 ml of the sodium sulfate-acetate mixture used for crystallizing serum albumin, no crystals form. Instead all the protein immediately precipitates amor- phously, and this precipitate does not dissolve when the salt solution is diluted with an equal volume of water. The albumin dissolved by placing crystals previously heated at 80° in the pH 9.2 borate buffer has the characteristic properties of a denatured protein. Denaturation is not caused by the pH 9.2 buffer, for if this buffer is added to native, unheated serum albumin, there is no difficulty in crystallizing the albumin and subsequently dissolving the crystals in water.

It is clear, then, that the heated crystals of serum albumin that are insoluble in water are crystals of denatured protein. Denaturation does not destroy the crystal pattern (although crystallographic analysis will probably show that it has been changed) but once the denatured albumin molecules are released from their confinement within the crystal by being dissolved in a pH 9.2 buffer it is impossible to replace them in the ordered pattern characteristic of a crystal of native protein. It is possible to obtain crystals of denatured protein by denaturing a protein while it is in the crystalline state, but it does not seem to be possible to crystallize a denatured protein.

¹A. E. Mirsky and L. Pauling, *Proc. Nat. Acad. Sci.*, 22: 439, 1936.

SULFHYDRYL GROUPS OF EGG ALBUMIN IN DIFFERENT DENATURING AGENTS

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When egg albumin is denatured there occurs a striking change in its SH groups. This change provides a clue to an understanding of the change in configuration of the egg albumin molecule that takes place during denaturation. A protein can be denatured by many different agents. This is a well known and important characteristic of protein denaturation. In the present investigation our purpose is to discover whether different denaturing agents liberate the same or different numbers of SH groups in egg albumin.

The change in SH groups is readily observed. Native egg albumin does not give a color reaction with nitroprusside and does not reduce ferricyanide; denatured egg albumin gives the color with nitroprusside characteristic of SH groups and immediately reduces ferricyanide (4, 17). This change in the SH groups of egg albumin is an example of the general rule that certain groups in a protein become reactive as a result of denaturation. Other groups, in addition to sulfhydryl, become reactive when egg albumin is denatured. Some of these groups also reduce ferricyanide, though they require a more alkaline medium for their activity than do SH groups. These reducing groups are probably the phenolic groups of tyrosine (20). The SH groups of denatured egg albumin react with iodoacetate (17). Other groups of denatured albumin react with iodoacetate (22). Some of these groups, as yet unidentified, do not react with iodoacetate while the protein is native. There are proteins (in striated muscle and the crystalline lens of the eye) in which, unlike egg albumin, some SH groups are reactive even while the proteins are in the native state. Even while native, these proteins give a color reaction with nitroprusside and reduce ferricyanide (19). In these proteins denaturation produces a marked increase in the number of reactive SH groups. In other proteins (the serum proteins of the horse, for example) no SH groups are detectable either before or after denaturation.¹ But in both native and denatured serum proteins disulfide (S—S)

¹ Greenstein found that horse serum albumin gives a nitroprusside reaction in presence of a high concentration of guanidine hydrochloride (10, 11). I can confirm this observa-

groups can be shown to be present and they can be estimated after being reduced to SH groups (18). It is then found that there is a larger number of reactive S—S groups in the denatured than in the native serum proteins. The method used for estimating S—S groups in the serum proteins was subsequently used to estimate S—S groups in insulin (23) and lactalbumin (12). Of all the instances of reactive groups appearing in proteins after denaturation the occurrence of reactive SH groups in egg albumin is an example that presents several advantages for investigation: SH groups can be estimated with precision, and the complete absence of reactive groups in native egg albumin makes the increase in number on denaturation especially striking.

The present investigation deals with the effects of three different denaturing agents on the SH groups of egg albumin. The three denaturing agents are urea, guanidine hydrochloride, and the synthetic detergent, Duponol P. C. (a mixture of the C₁₀–C₁₈ compounds of the series CH₃(CH₂)_nCH₂OSO₃Na) (1, 3, 8, 21). In each instance the denatured protein remains in solution while the denaturing agent is present.

Method

Protein SH groups are estimated by means of their reaction with ferricyanide, as a result of which they are oxidized to S—S groups and ferrocyanide is formed.



An excess of ferricyanide is added and the quantity of ferrocyanide formed is estimated. This is done by adding ferric sulfate which reacts with ferrocyanide to form Prussian blue which is estimated with a photoelectric colorimeter of the Evelyn type. The intensity of the blue color is a measure of the number of active, protein SH groups. Before adding ferric sulfate it is necessary either to remove the protein or to add some reagent that will keep protein in solution even in presence of ferric sulfate. Both procedures are followed.

For relatively simple SH compounds, such as cysteine and glutathione, the reaction with ferricyanide proceeds stoichiometrically. There is no difficulty in titrating the SH groups of glutathione with ferricyanide (16). The titration is simple and accurate, with a sharp end-point. Ferricyanide

tion; a definite but not intense reaction is obtained. Estimation of the number of SH groups shows that less than 0.1 per cent is present, hardly a significant quantity.

has already been used to estimate the SH groups of a protein-denatured globin (20). It was at that time observed that "Whereas the oxidation of SH to S—S by ferricyanide is a definite reaction under suitable conditions ($2 \text{ SH} + 2 \text{ ferricyanide} = 1 \text{ S—S} + 2 \text{ ferrocyanide}$) the reaction of the other (reducing) groups (of a protein) with ferricyanide is not so definite. The greater the ferricyanide concentration and the longer the time of reaction, the more oxidation by ferricyanide takes place." At that time ferricyanide was not used to estimate the SH groups of denatured egg albumin. A more cumbersome procedure was followed. Conditions under which the reaction between ferricyanide and the SH groups of denatured egg albumin is precise and definite have now been found; the protein should be dissolved in approximately neutral solutions of urea, guanidine hydrochloride, or Duponol. Under these conditions the reaction goes with great speed. It is completed in less than 1 minute; no more ferricyanide is reduced in 60 minutes than in 1 minute. Nor within wide limits do the concentration of ferricyanide or temperature affect the quantity of ferricyanide reduced. These observations suggest that in the reaction between ferricyanide and egg albumin in neutral solutions of urea, guanidine hydrochloride, or Duponol only the SH groups of the albumin reduce ferricyanide.²

There are, as mentioned above, other reducing groups in denatured egg albumin, but those that have been investigated reduce ferricyanide in a slightly alkaline medium only. Furthermore, the reaction of these non-SH groups with ferricyanide is sluggish, there being no definite end-point, and the quantity of ferricyanide reduced depends upon the concentration of ferricyanide present. These groups, then, do not take part in the clearly defined reaction between egg albumin and ferricyanide in a neutral medium.

That the groups of denatured egg albumin which reduce ferricyanide in neutral medium appear to be SH groups and nothing but SH groups can be shown by using the nitroprusside test, especially in conjunction with certain reagents that combine with SH groups. This test in a protein may be considered to be specific for SH groups, for no other groups in a protein are known to give a color reaction with nitroprusside. It is possible that there

² After most of the experiments on egg albumin in solutions of urea and guanidine described in this paper were completed, Anson discovered the effect of Duponol (1). It was then that Duponol was used in the experiments reported in this paper. Anson found that the amount of ferricyanide reduced by denatured egg albumin in Duponol P C solution is within wide limits independent of the concentration of ferricyanide and the time, temperature, and pH of the reaction. The similar observations on egg albumin in solutions of urea and guanidine hydrochloride described in this paper had already been independently made.

are SH groups which do not give a nitroprusside test and which might react with some reagent other than ferricyanide. A detailed comparison under many different conditions of the color reaction of egg albumin with nitroprusside and the reducing reaction with ferricyanide shows a close correlation between these two reactions. When egg albumin reduces ferricyanide in neutral medium it gives a color with nitroprusside, and when it does not reduce ferricyanide it fails to give a color with nitroprusside. A number of examples of this correlation may be cited:

1. Native egg albumin does not give a color test with nitroprusside; nor does it reduce ferricyanide.

2. When egg albumin is denatured by urea, guanidine hydrochloride, Duponol, or any other agent, it gives a nitroprusside test and also reduces ferricyanide. After the reaction with ferricyanide is completed the albumin no longer gives a nitroprusside test.

3. Heat coagulated egg albumin is treated with ferricyanide and the excess ferricyanide is washed away when the reaction appears to be ended. The albumin no longer gives a test with nitroprusside. Guanidine hydrochloride is then added to the albumin. The albumin now gives a color reaction with nitroprusside and also reduces ferricyanide.

4. Egg albumin denatured by urea is oxidized with ferricyanide and the excess ferricyanide is removed. The albumin no longer gives a test with nitroprusside. The albumin is now treated with guanidine hydrochloride. Neither a nitroprusside test nor reducing action with ferricyanide is observed.

5. When guanidine hydrochloride is added to egg albumin solutions in the pH range from 5.8 to 7.8 precisely the same quantities of ferricyanide are reduced and in no case is a nitroprusside reaction observed when the excess ferricyanide is removed. When guanidine hydrochloride is added to albumin at pH 4.4, 21 per cent less ferricyanide is reduced. Now when the excess ferricyanide is removed and the albumin is tested with nitroprusside in presence of guanidine hydrochloride a slight color reaction is observed. This albumin, brought to pH 7.0, reduces ferricyanide in presence of guanidine hydrochloride. It reduces 16 per cent of the quantity it would have reduced if it had not previously reacted with ferricyanide at pH 4.4.

6. The SH groups of denatured egg albumin react with iodoacetate and iodoacetamide. Egg albumin, so treated, no longer gives a nitroprusside test; nor does it reduce ferricyanide.

7. SH groups of denatured egg albumin, like other SH groups, react with mercuric chloride. After the reaction the albumin neither gives a nitroprusside test nor reduces ferricyanide.

The close correlation between the nitroprusside test and the tendency to reduce ferricyanide makes it unlikely that there are any other groups in egg albumin in addition to SH which reduce ferricyanide in a neutral medium. It is also unlikely, quite apart from the nitroprusside test, that there are any groups other than sulfhydryl in egg albumin which combine with iodoacetate and mercuric chloride and which also reduce ferricyanide in neutral medium. And yet it should be recognized that the existence of such groups has not been *completely* excluded. Ferricyanide certainly reacts with all SH groups giving a nitroprusside test. But it is possible that there are a few non-SH groups in a protein that react with ferricyanide in neutral medium. In a protein the reactive range of other reducing groups may overlap to a slight extent with the range of activity of SH groups. With this reservation the quantity of ferrocyanide formed can be taken as a measure of the number of reacting SH groups. It remains to be shown that none of the ferrocyanide formed in the reaction between protein and ferricyanide is lost in those cases in which the protein is removed before estimating ferrocyanide. And, in fact, none is lost, for it is found that ferrocyanide added before removing protein is completely recovered when the protein is subsequently removed.

Denaturation

A protein is said to be denatured when it is insoluble in a medium in which it is soluble while still native. Egg albumin denatured by heat is insoluble in water at the isoelectric point, pH 4.7—a medium in which native egg albumin is soluble. The egg albumin in urea, guanidine hydrochloride, or Duponol, which reduces ferricyanide is denatured. It is soluble at the isoelectric point in the presence of urea, guanidine hydrochloride, or Duponol, but when these denaturing agents are removed or diluted with water the protein is found to be insoluble.

Experiments on egg albumin in urea solutions show clearly that liberation of SH groups and formation of insoluble protein are integral parts of the same process. To liberate the maximum number of SH groups 1 gram of urea is added to each 1 cc. of albumin solution. After standing for 60 minutes the albumin reduces no more ferricyanide than it does after standing for only 30 minutes; and within 30 minutes all of the albumin is denatured. This can be shown by diluting the urea solution with water, adjusting the pH to 4.7, and adding one quarter of the volume of saturated $(\text{NH}_4)_2\text{SO}_4$ (much less than is needed to precipitate native egg albumin). No protein is left in solution. The correlation between liberation of SH

groups and formation of insoluble protein becomes more apparent when insufficient urea is added to liberate all the SH groups. If the number of SH groups liberated is estimated at different intervals of time after adding urea, it is found that with advancing time more and more groups are liberated so that even after 3 hours in urea and ferricyanide no end-point is reached. Tests for presence of insoluble egg albumin (made by adding water, pH 4.7 acetate buffer, and saturated $(\text{NH}_4)_2\text{SO}_4$) show that part of the albumin is insoluble, but that some remains soluble. The soluble and insoluble fractions are separated from each other and both are washed free of ferricyanide. In the insoluble fraction there are no more SH groups; this fraction does not give a reaction with nitroprusside even in presence of guanidine hydrochloride. The soluble fraction still contains SH groups; if this albumin is denatured by adding guanidine hydrochloride an intense nitroprusside reaction is observed. Estimation of the number of SH groups in the albumin of the soluble fraction after denaturation by addition of Duponol shows that per milligram of protein there is the same number of groups as in egg albumin not previously treated with urea and ferricyanide. That fraction of egg albumin in urea and ferricyanide which becomes insoluble (when tested under certain clearly defined conditions) has all of its SH groups liberated, whereas albumin that still is soluble has none of its SH groups liberated. Denaturation of egg albumin by urea is a discontinuous process. A given molecule of protein is either native or denatured. Denaturation of egg albumin by some other agents, it will be shown in other papers, is also an all-or-none process.

When enough guanidine hydrochloride is added to egg albumin to liberate the maximum number of SH groups all of the albumin loses its solubility, when tested in the same manner as in the experiments with urea. In experiments with Duponol a different procedure is followed to demonstrate the altered solubility of albumin. The solution of albumin in Duponol is dialyzed against water for a long time to remove as much Duponol as possible. To the clear dialysate is added 1/10 its volume of saturated ammonium sulfate. This suffices to precipitate all the protein in solution, indicating that Duponol, as well as urea and guanidine, alters the solubility of egg albumin.

RESULTS

Since the only amino acid known to have SH groups is cysteine, the SH groups of egg albumin are considered to be part of cysteine, and are accordingly expressed as percentage of cysteine. The results are reproducible to within ± 5 per cent.

It can be seen from Table I that the quantities of ferricyanide reduced by egg albumin in urea, guanidine hydrochloride, and Duponol are about

TABLE I

SH Groups of Egg Albumin Expressed As Per Cent of Cysteine, Denatured by Urea, Guanidine Hydrochloride, and Duponol

| Albumin preparation No. | Albumin solution | Albumin | Ferricyanide added | Volume after adding buffer ferricyanide | pH | Denaturing agent added | Temperature | Time of reaction | Quantity ferricyanide formed | SH groups | Cysteine |
|-------------------------|------------------|---------|--------------------|-----------------------------------------|-----|---------------------------|-------------|------------------|------------------------------|------------|----------|
| | cc. | mg. | mm | cc. | | mg. | °C. | min. | mm | mg. | per cent |
| | | | | | | (Urea) | | | | (Cysteine) | |
| I | 0.25 | 18.5 | 0.005 | 0.350 | 6.7 | 350 | 37.5 | 30 | 0.00145 | 17.5 | 0.95 |
| | | | | | | | 37.5 | 15 | 0.00144 | 17.3 | 0.94 |
| | | | | | | | 25.0 | 30 | 0.00144 | 17.3 | 0.94 |
| | | | | | | | 25.0 | 30 | 0.00140 | 16.8 | 0.91* |
| | | | | | | (Guanidine hydrochloride) | | | | | |
| I | 0.25 | 18.5 | 0.005 | 0.350 | 6.7 | 300 | 25.0 | 30 | 0.00142 | 17.0 | 0.92 |
| | | | 0.025 | | | | | | 0.00144 | 17.3 | 0.94 |
| | | | 0.005 | | | | | 15 | 0.00144 | 17.3 | 0.94 |
| | | | 0.005 | | | | 0 | 15 | 0.00145 | 17.5 | 0.95 |
| | | | 0.010 | | 7.8 | | 25.0 | 30 | 0.00146 | 17.6 | 0.95 |
| | | | 0.010 | | 5.8 | | 25.0 | 30 | 0.00143 | 17.2 | 0.93 |
| | | | 0.010 | | 4.4 | | 25.0 | | 0.00114 | 13.7 | 0.74 |
| | | | | | | (Duponol) | | | | | |
| I | 0.25 | 18.5 | 0.005 | 1.85 | 6.7 | 50 | 37.5 | 10 | 0.00153 | 18.35 | 0.99 |
| | 0.25 | 18.5 | 0.005 | 1.85 | 6.7 | | 37.5 | 10 | 0.00148 | 17.8 | 0.96 |
| II | 0.20 | 20.8 | 0.005 | 1.85 | 6.7 | | 37.5 | 10 | 0.00171 | 20.6 | 0.99 |
| | 0.20 | 20.8 | 0.005 | 1.85 | 6.7 | | 37.5 | 10 | 0.00168 | 20.2 | 0.97 |
| III | 0.35 | 17.5 | 0.005 | 1.85 | 6.7 | | 37.5 | 10 | 0.00137 | 16.45 | 0.94 |
| | 0.35 | 17.5 | 0.005 | 1.85 | 6.7 | | 37.5 | 10 | 0.00143 | 17.15 | 0.98 |

* In this experiment the albumin was precipitated with tungstic acid before estimation of ferrocyanide. In the other experiments with urea, Duponol was added to prevent precipitation of protein during the ferrocyanide estimation.

the same. Guanidine hydrochloride and Duponol do not liberate any SH groups not liberated by urea, as the following experiment shows: Egg albumin in urea is oxidized by ferricyanide. After the urea and ferricyanide have been washed away, the albumin fails to give a reaction with nitroprusside or to reduce ferricyanide when placed in either guanidine hydrochloride or Duponol.

Three different preparations of crystalline egg albumin were used in the experiments described in this paper. For most of the experiments a single preparation was used. Two other preparations were made to learn whether different samples of egg albumin contain the same number of SH groups when denatured. Of these preparations one (preparation number II of Table I) was made by the method of Kekwick and Cannan (13) and the other (preparation number III of Table I) by La Rosa's method. Preparation III was investigated in the electrophoresis apparatus of Tiselius by Dr. Longworth. No appreciable quantity of any protein constituent of egg white except egg albumin could be detected. All three preparations of albumin when denatured by Duponol were found to contain the same number of SH groups.

There have been several investigations of the SH groups of egg albumin denatured by urea and guanidine hydrochloride. Rosner estimated SH groups by means of their reaction with iodoacetate (22). He found 0.87 per cent in egg albumin denatured by urea. This is about 10 per cent lower than the result obtained by the reaction with ferricyanide—an entirely different method. Greenstein estimated SH groups by titrating them with porphyrindin, a powerful oxidizing agent (8). For egg albumin in urea he obtained 1.00 per cent SH and for albumin in guanidine hydrochloride 1.28 per cent. The results given by the porphyrindin and ferricyanide methods for the SH groups of albumin in urea are in good agreement. In guanidine hydrochloride the two methods do not agree; in this medium porphyrindin titration gives a much higher value. What seems especially significant in the porphyrindin titrations, and this point has been emphasized by Greenstein, is that different denaturing agents, such as urea and guanidine hydrochloride, liberate different numbers of SH groups. This apparent difference in SH groups seems to be due to a defect in method; porphyrindin may react with reducing groups other than SH in a denatured protein. This possibility was recognized by Kuhn and Desnuelle who first used porphyrindin for titrating protein SH groups (14). They accordingly carried out the reaction at 0° and in an especially careful manner. They placed some confidence in their results on heat coagulated egg albumin because they were in agreement with results obtained by other methods (17, 24). To Greenstein it seemed "hardly probable that the dye (porphyrindin) would react with other types of reducing groups in the protein. Such groups, involving tyrosine and tryptophane radicals, as Mirsky and Anson point out, only begin to make their presence felt at pH 10 and, moreover, react very slowly with ferricyanide and not at all with cystine or phosphotungstate. It is certain in any case that they would not exhibit

a nitroprusside reaction." The fact that the non-SH reducing groups of denatured egg albumin do not reduce ferricyanide at pH 7.0 but require a more alkaline medium does not mean that these groups will fail to reduce porphyrindin, a more powerful oxidant than ferricyanide, at pH 7.0 (and in the presence of guanidine hydrochloride). Indeed Kuhn and Desnuelle point out that porphyrexid (closely related to porphyrindin) oxidizes thiamin to thiochrome in neutral solution while potassium ferricyanide requires an alkaline solution for the same oxidation.³ And although it may be certain that the non-SH reducing groups of denatured egg albumin "would not exhibit a nitroprusside reaction" this does not prove that they do not react with porphyrindin in presence of guanidine hydrochloride. Greenstein showed that the groups of egg albumin exhibiting a nitroprusside reaction reduce porphyrindin; but he did not show that the groups not exhibiting a nitroprusside reaction do not reduce porphyrindin. Denatured excelsin, he observed, neither gives a nitroprusside reaction nor reduces porphyrindin (9). On the other hand crystalline papain reduces more porphyrindin than can be accounted for by its sulfur content (5). There is then some doubt concerning the estimation of protein SH groups by titration with porphyrindin.⁴ The difference in the quantities of porphyrindin reduced by egg albumin in urea and in guanidine hydrochloride (a difference of 28 per cent) is not due to there being an increased liberation of SH groups in guanidine hydrochloride, for if this were so, egg albumin in urea that had been oxidized with ferricyanide would subsequently give a nitroprusside reaction when dissolved in guanidine hydrochloride—and, as stated above, a nitroprusside test is not obtained under these conditions.

Anson finds the same number of SH groups (equivalent to a cysteine content of 1.2 per cent) present in egg albumin denatured by guanidine hydrochloride and Duponol (2).

To explain why active SH groups appear in egg albumin when it is denatured, measurements of SH groups must be combined with other kinds of information about the protein. Such investigations have already been carried out and will be described in another paper. The significance of the measurements made in the present investigation will then become clear, as will also the conclusion that the same number of active SH groups is present in egg albumin denatured by urea, guanidine hydrochloride, or Duponol.

³ "Mit Porphyrexid lässt sich Aneurin in neutraler Lösung zu Thiochrom oxydieren, was sonst nur noch mit Kaliumferricyanid in alkalischer Lösung gelingt."

⁴ The validity of SH estimations in proteins by titration with porphyrindin has also been questioned by Brand and Kassell (6).

SUMMARY

1. The reaction between ferricyanide and egg albumin in solutions of urea, guanidine hydrochloride, and Duponol has been investigated.

2. In neutral medium ferricyanide oxidizes all the SH groups of egg albumin that give a color reaction with nitroprusside. In neutral medium ferricyanide appears to react only with the SH groups of egg albumin. The quantity of ferrocyanide formed can accordingly be considered the equivalent of the number of SH groups in egg albumin detectable with nitroprusside.

3. In solutions of urea, guanidine hydrochloride, and Duponol sufficiently concentrated so that all the egg albumin present is denatured, the same number of SH groups are found—equivalent to a cysteine content of 0.96 per cent.

4. In denaturation of egg albumin loss of solubility (solubility not in presence of the denaturing agent, but solubility examined in water at the isoelectric point) and appearance of reactive SH groups are integral parts of the same process. As denaturation proceeds in urea, SH groups are liberated only in the egg albumin with altered solubility and in this albumin the maximum number of SH groups is liberated. In a molecule of egg albumin either all of its SH groups that give a test with nitroprusside are liberated or none of them are.

EXPERIMENTAL

The egg albumin used in most of these experiments was prepared by La Rosa's method and then recrystallized three times (15). The albumin used in one experiment was prepared by the method of Kekwick and Cannan (13). Before being used a sample of egg albumin was dialyzed in a rocking dialyzer until completely free of ammonium sulfate. Concentration of egg albumin was then determined by drying to constant weight at 105°. The albumin solution was stored in the cold without preservative for the few days during which it was used. Solutions of ferricyanide were used within 3 or 4 days after being made up and during this time were kept in the dark at 1°C.

Deproteinization

Before estimating the quantity of ferrocyanide formed in the egg albumin solution it is necessary (except in the presence of Duponol) to remove the protein. This is done with tungstic acid. A 10 per cent stock solution of sodium tungstate is acidified whenever tungstic acid is needed. To 1.0 cc. of the sodium tungstate solution are added 40 cc. water, 0.70 cc. of 1 N H₂SO₄, and enough water to bring the volume to 50 cc. In presence of Duponol, ferric sulfate does not precipitate protein.

Estimation of Ferrocyanide as Prussian Blue

Prussian blue is formed when ferric sulfate is added to an acidified solution of ferrocyanide. There is a tendency for Prussian blue to precipitate. This can be prevented by adding gum ghatti (7). It is convenient to prepare a solution of ferric sulfate in gum ghatti, as described by Folin and Malmros. To 5 cc. of deproteinized (or Duponol containing) solution are added 0.05 cc. of 0.2 M potassium ferricyanide, 1 cc. of ferric sulfate-gum ghatti and then after 5 minutes, 6.5 cc. water. After standing 5 more minutes Prussian blue is estimated in a photoelectric colorimeter of the Evelyn type, using a red filter (Corning No. 241). A red filter is used because the ferricyanide present does not absorb red light to a significant extent, and ferricyanide is present since an excess is added to the albumin. Still more ferricyanide is added at the time of Prussian blue formation because it was found that when minute quantities of ferrocyanide are being estimated, the amount of Prussian blue formed (in the time interval employed) is increased if ferricyanide is present. With the quantity of ferricyanide added the maximum amount of Prussian blue is formed.

To establish a relationship between intensity of color and quantity of ferrocyanide, Prussian blue is formed in solutions containing known quantities of ferrocyanide. In analytical chemistry standard solutions of ferrocyanide are ordinarily considered to be stable. The solutions required in the present experiments are far more dilute (0.0002 M) than those usually used and these dilute solutions of potassium ferrocyanide are not stable. They must be prepared from more concentrated (0.1 M) stock solutions or from solid potassium ferrocyanide whenever an experiment is done. Prussian blue is formed from known quantities of ferrocyanide under precisely the same conditions as when unknown quantities are present. The 5 cc. of solution, to which 1 cc. of ferric sulfate-gum ghatti subsequently is added, contains between 0.50 and 2.5 cc. of 0.0002 M ferrocyanide. Also included in the 5 cc. of solution are 0.05 cc. 1 N H_2SO_4 , 0.05 cc. 0.2 M K ferricyanide, and 2.5 cc. tungstic acid. Of these reagents only ferricyanide influences the color intensity of the blue solution finally obtained. Urea, Duponol, and guanidine hydrochloride have also been added to solutions containing known quantities of ferrocyanide. The quantity of Duponol present in the experiments with egg albumin does not affect the intensity of color when Prussian blue is formed. Urea and guanidine hydrochloride do affect the intensity of color, and it is accordingly necessary to have urea and guanidine hydrochloride in the standard ferrocyanide solutions when these reagents are added to egg albumin.

Reactions between Egg Albumin and Ferricyanide

1. *In Urea.*—To 0.25 cc. of a 7 per cent albumin solution are added 0.05 cc. 1 M KH_2PO_4 - K_2HPO_4 pH 6.7 buffer, 0.05 cc. 0.1 M K ferricyanide, and 350 mg. of urea (100 mg. urea added to each 0.10 cc. of albumin containing solution). A number of these solutions are prepared and kept for various periods of time, some at 25°C., others at 37.5°. After the reaction with ferricyanide, to each solution are added 10 cc. tungstic acid, 0.4 cc. 1 N H_2SO_4 , and enough water to bring the volume to 20 cc. The mixture is filtered and 5 cc. of the clear filtrate are taken for Prussian blue formation. Instead of removing egg albumin by precipitation with tungstic acid, the albumin can be left in

solution if Duponol is added for this prevents protein precipitation when ferric sulfate is added. After the reaction between albumin and ferricyanide, 15 cc. of water and 0.4 cc. 1 N H_2SO_4 are added. As the acid is mixed with the protein solution a fine precipitate appears. This clears up at once when 0.5 cc. of a 10 per cent Duponol solution is added. The solution is brought to a volume of 20 cc. by addition of water. When Duponol is added to a strongly acid mixture of egg albumin and ferricyanide, no reaction between protein SH groups and ferricyanide occurs.

Effect of Iodoacetamide and Mercuric Chloride.—To 1 cc. of albumin are added 0.3 cc. phosphate buffer, 0.65 cc. H_2O , 25 mg. iodoacetamide, and 2.0 gm. urea. After standing for an hour at 25°C., 0.5 cc. ferricyanide is added and 30 minutes later the albumin is precipitated with tungstic acid. No Prussian blue forms in the filtrate when ferric sulfate is added. Iodoacetamide does not interfere with Prussian blue formation when it is present in a ferrocyanide solution to which ferric sulfate is added. In another experiment 0.2 cc. of 0.1 M $HgCl_2$ is added instead of iodoacetamide. No Prussian blue is found in this case either.

Albumin in Urea Oxidized by Ferricyanide and Subsequently Treated with Guanidine Hydrochloride or Duponol

To 1.25 cc. albumin are added 0.5 cc. phosphate buffer, 0.25 cc. ferricyanide, and 2 gm. urea. After 30 minutes the albumin is precipitated with tungstic acid and the suspension is centrifuged. The protein precipitate is washed with tungstic acid until it is colorless. To the precipitate are added 0.4 cc. 1 M K_2HPO_4 , 1 cc. of a 10 per cent Duponol solution, 0.1 cc. ferricyanide, and water to bring the volume to 15 cc. At 37.5° this mixture forms a clear solution. After 20 minutes the solution is acidified with 1 N H_2SO_4 and diluted with water to 20 cc. Of this solution 5 cc. are taken to test for Prussian blue formation, but no color appears. In another experiment guanidine hydrochloride instead of Duponol is added to the tungstic acid precipitate of albumin. The precipitate is packed down hard in the centrifuge and to it are added 0.3 cc. 1 M K_2HPO_4 and 300 mg. guanidine hydrochloride. A small part of the mixture is tested with nitroprusside. No color is observed. To the rest of the albumin-guanidine hydrochloride mixture is added 0.05 cc. ferricyanide. After 30 minutes at 25°, 10 cc. of tungstic acid, 0.5 cc. H_2SO_4 , and water are added to bring the volume to 20 cc. No Prussian blue appears in the filtrate when ferric sulfate is added.

Test for Completeness of Denaturation.—To 1 cc. of the albumin solution 1 gm. of urea is added. After the solution has stood at 25° for 30 minutes it is diluted to 10 cc. To 5 cc. are added 0.25 cc. of a 2 M pH 4.7 acetate buffer and 1.25 cc. saturated $(NH_4)_2SO_4$. The suspension is filtered. Only a slight haze appears in the filtrate when trichloroacetic acid is added.

Oxidation of Albumin in Insufficient Urea to Produce Complete Denaturation

To 15.5 cc. of albumin solution are added 1.5 cc. of phosphate buffer, 3.0 cc. of 0.1 M ferricyanide, and 13 gm. of urea. The solution remains at 25° for 20 minutes. A heavy precipitate is formed when 20 cc. of a pH 4.7, 1 M acetate buffer are added and with this

suspension 10 cc. of a saturated ammonium sulfate solution are mixed. A clear supernatant is obtained after centrifuging. The supernatant solution is dialyzed at 1° in a rocking dialyzer against distilled water for 24 hours. It is then completely free of ferricyanide, but somewhat turbid. This fluid is filtered and the protein content of the clear filtrate is determined by drying in an oven at 105°. Each cc. contains 8.52 mg. of protein. Since there are 64 cc. of this solution (561.3 mg. in all) and since the 15.5 cc. of albumin solution used at the beginning of the experiment contained 70.2 mg. per cc. (1088 mg. in all), somewhat less than 50 per cent of the albumin originally present was denatured by urea. SH groups in the dialyzed egg albumin solution are estimated by adding to 2 cc. of the solution 0.05 cc. phosphate buffer, 0.05 cc. of 0.1 M ferricyanide, and 0.5 cc. of a 10 per cent Duponol solution and then proceeding as described below. The albumin precipitated from urea solution by adding acetate buffer is washed with a $\frac{1}{2}$ saturated $(\text{NH}_4)_2\text{SO}_4$ solution, removing the washings by centrifuging, until the protein precipitate is free of the yellow color of ferricyanide and the washings contain no protein precipitable with trichloroacetic acid. The protein precipitate is then tested for SH groups with nitroprusside and ammonium hydroxide in the presence of guanidine hydrochloride. The test is negative.

2. *In Guanidine Hydrochloride.*—300 mg. guanidine hydrochloride are dissolved in 0.25 cc. albumin. The solution remains at 25° for 30 minutes and then to it are added 0.05 cc. of 1 M pH 6.8 potassium phosphate buffer and 0.05 cc. of 0.1 M ferricyanide. After the reaction with ferricyanide the protein is precipitated by adding 10 cc. tungstic acid and 0.4 cc. of 1 N H_2SO_4 . The suspension is diluted with water to a volume of 20 cc., shaken, and filtered. Of the filtrate 5 cc. are taken for Prussian blue formation. The conditions of the reaction between ferricyanide and egg albumin in guanidine hydrochloride are varied: the temperature is either 25 or 37.5°; tungstic acid is added to the solution at intervals varying from 5 to 80 minutes after mixing ferricyanide with the albumin; the concentration of ferricyanide added is either 0.1 M or 0.5 M; the quantity of guanidine hydrochloride added is either 200 or 300 mg.; ferricyanide, phosphate buffer, and albumin are mixed together before adding guanidine hydrochloride. None of these variations affects the quantity of ferricyanide reduced. The phosphate buffer used can vary in pH from 6.1 to 7.3, or even 1 M K_2HPO_4 can be used, without affecting the reaction, but if an acetate buffer of pH 4.7 is used, less ferricyanide is reduced.

Effects of Iodoacetamide and Mercuric Chloride.—After dissolving 300 mg. of guanidine hydrochloride in 0.25 cc. of albumin, 0.05 cc. of phosphate buffer is added. In one experiment 20 mg. of iodoacetamide are added to this solution and in another experiment 0.05 cc. of 0.1 M HgCl_2 is added. After standing for an hour at 25°C., 0.05 cc. of ferricyanide is added to each albumin-guanidine hydrochloride mixture and after another hour tungstic acid is added. No blue color appears in the filtrate when ferric sulfate is added.

Recovery of Ferrocyanoide Added to Egg Albumin in Guanidine Hydrochloride.—The egg albumin in 0.25 cc. is oxidized by ferricyanide in the presence of guanidine hydrochloride and then precipitated by tungstic acid. The precipitated albumin is separated by centrifuging, washed free of ferricyanide with tungstic acid, and then washed with 0.1 M pH 6.8 phosphate. To the precipitated albumin are added 300 mg. guanidine hydrochloride, 0.05 cc. of 1 M phosphate, 0.05 cc. of ferricyanide, and finally 1 cc. of 0.001 M ferrocyanoide. After standing for 10 minutes, tungstic acid and water to bring the volume to 20 cc. are

added and the suspension is filtered. The quantity of Prussian blue formed by the ferrocyanide in 5 cc. of the filtrate is compared with the quantity formed in 5 cc. of a control solution made by adding 1 cc. of ferrocyanide to 10 cc. of tungstic acid, 0.05 cc. of ferricyanide, and 8.5 cc. of water. The galvanometer readings of the colorimeter were practically identical for the two Prussian blue solutions showing that all of the ferrocyanide added to the egg albumin was recovered.

Completeness of Denaturation in guanidine hydrochloride was demonstrated as it was in urea: 300 mg. of guanidine hydrochloride were dissolved in 0.25 cc. of albumin. After standing for 15 minutes, 4 cc. of water, 0.25 cc. of a 2 M pH 4.7-acetate buffer, and 1 cc. of saturated $(\text{NH}_4)_2\text{SO}_4$ are added. The precipitate is filtered off. Trichloroacetic acid is added to the precipitate. No sign of a protein precipitate is detectable.

3. *In Duponol*.—To 0.25 cc. of the albumin solution are added 0.05 cc. of pH 6.8 1 M phosphate buffer, 2 cc. of water, 0.5 cc. of a 10 per cent Duponol solution, and 0.05 cc. of ferricyanide. The solution is kept at 37.5° for 10 minutes. It is then acidified by the addition of 0.4 cc. 1 N H_2SO_4 and diluted to 20 cc. with water. 5 cc. of this solution are taken for Prussian blue formation.

Effects of Iodoacetamide and Mercuric Chloride.—To 1.5 cc. of the albumin solution are added 0.2 cc. of phosphate buffer, 0.5 cc. of Duponol, and either 25 mg. of iodoacetamide or 0.2 cc. of 0.1 M HgCl_2 . After 10 minutes at 37° 0.05 cc. of ferricyanide is added. The solution remains at 37.5° for 30 minutes before acidifying and diluting to 20 cc. No Prussian blue appears in the filtrate when ferric sulfate is added.

Completeness of Denaturation.—To 2 cc. of albumin solution are added 10 cc. of water and 4 cc. of Duponol. The solution is placed in a cellophane tube and dialyzed against distilled water in a rocking dialyzer for 36 hours at 37.5°. At the end of this time the albumin solution remains clear. To 3 cc. of the solution is added 0.05 cc. of a saturated ammonium sulfate solution. A heavy precipitate forms. After filtration 0.5 cc. of 50 per cent trichloroacetic acid is added to the clear filtrate. No turbidity appears.

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SULFHYDRYL GROUPS IN FILMS OF EGG ALBUMIN

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The shape of the protein molecule changes completely when a protein spreads to form a unimolecular film at an interface. The egg albumin molecule, for example, may be considered to be an ellipsoid in solution with a major axis of 91 A.u. and a minor axis of 32 A.u. (10). In the film it flattens out to a thickness of only 8 A.u. (4). There are groups in native soluble egg albumin which do not react with certain reagents. The SH groups of denatured egg albumin give a color reaction with nitroprusside, reduce ferricyanide, and react with iodoacetate in much the same manner as do the SH groups of cysteine (8). In native egg albumin the SH groups do not show these reactions. The present investigation deals with the activity of the SH groups in thin films of egg albumin. Information concerning the SH groups in the protein film throws light on two problems:

1. In a large molecule such as that of egg albumin, 32 A.u. in diameter, many atoms will occupy inner positions in the elaborate structure. The arrangement of the peripheral groups of atoms may be so compact as to constitute a barrier preventing contact between inner groups of atoms and many of the substances dissolved in the medium in which the protein itself is dissolved. There would, according to this view, be an *interior* of the protein molecule which is definitely less accessible to many reagents than the periphery of the molecule. The structure of the protein molecule may, on the other hand, be so loosely knit that there is no significant distinction between peripheral and internal groups, at least in so far as concerns accessibility to reagents in the surrounding media. When egg albumin spreads at an interface the film of protein is so thin that there can hardly be said to be an "interior" of the molecule. In the film all groups are on the periphery. The protein molecule in forming a film may be said to unfold. If groups inactive in the native egg albumin molecule become active when a film is formed, this is an indication that the groups have become exposed due to the unfolding process—an indication that the peripheral groups of the native, unchanged, egg albumin molecule act as a barrier between reagents in the surrounding solution and groups in the interior

of the protein molecule. Evidence for the existence of such an interior is provided by investigation of SH groups in films of egg albumin.

2. The protein in the film is insoluble. If the film of protein is rolled up and immersed in the medium in which it had been dissolved, it no longer dissolves. In this respect it resembles protein that has been denatured by heat, alcohol, acid, alkali, urea, guanidine hydrochloride, and other agents, and it has indeed been suggested that the protein in the film is denatured (2, 9). One of the characteristics of denaturation is the appearance of active groups. In the denaturation of egg albumin the appearance of active SH groups and the formation of insoluble protein (insoluble in water at the isoelectric point) are integral parts of the same process. The appearance of these groups in the film of egg albumin would indicate that this protein too is denatured; that when a film is formed the same fundamental change in the protein occurs as when it is heated or dissolved in urea, guanidine hydrochloride, or Duponol. Estimation of SH groups in films of egg albumin does in fact provide an insight into the whole process of protein denaturation.

Method

SH groups in the films are estimated by their reaction with ferricyanide. It has been shown that in neutral medium the quantity of ferricyanide reduced by egg albumin may be taken as the equivalent of the number of active SH groups present (7). The quantity of ferrocyanide formed by the SH groups in a single protein film (of practicable area) would be less than can be estimated by methods now available. For each experiment a great many films must be used. These are easily prepared. At the surface of an egg albumin solution there is always a film of protein and when the film is removed another forms. By continually shaking an albumin solution, the films that form are constantly removed until eventually all the protein originally in solution is in the form of clumped together, insoluble films. In this way large quantities of "film protein" can be had. The reaction between ferricyanide and film protein can take place under two different conditions. In one procedure the ferricyanide is mixed with a certain quantity of clumped together film protein, previously prepared by shaking an albumin solution. The finely divided suspension is constantly agitated to promote mixing. In another procedure ferricyanide is added to the egg albumin solution before the film protein is prepared. Then while the solution is shaken the ferricyanide is on the spot to react with each film while it is still a surface film. As the ferricyanide-albumin solution is shaken, more and more films are formed. At any given time the reaction

may be stopped by adding tungstic acid. The quantity of ferrocyanide in the protein-free filtrate is then estimated. In another sample of albumin the quantity of insoluble protein formed at that time is estimated. In procedure I, ferricyanide reacts with albumin only after it has been removed from the surface; in procedure II, ferricyanide is able to react with albumin while it still is at the surface.

RESULTS AND DISCUSSION

It can be seen from Table I that when ferricyanide is present to react with the films of egg albumin while they are being formed (procedure II) the results are precise and definite. The quantity of ferrocyanide formed for each milligram of insoluble egg albumin is constant, although the time of reaction and the amounts of insoluble albumin vary considerably. The quantity of ferrocyanide formed per milligram of insoluble albumin is the same at pH 6.9 as at pH 7.4, but more than at pH 6.6. Less ferricyanide is reduced at pH 6.6 because some of the SH groups fail to take part in the reaction, as tests with nitroprusside demonstrate. The tests are made on insoluble egg albumin that has been washed free of soluble egg albumin and ferricyanide. When samples of precipitated egg albumin from experiments at pH 6.6, 6.9, and 7.4 are tested with nitroprusside and ammonium hydroxide, in no case is a color obtained. When the same tests are carried out in the presence of guanidine hydrochloride an intense color appears in the albumin from the experiment at pH 6.6 and only just detectable colors in the albumin from the experiments at pH 6.9 and 7.4. It is characteristic of SH groups in general that the more acid the solution in which they are placed the less readily do they react. It is also characteristic of an SH group that its hydrogen is readily displaced by mercury. If mercuric chloride is added to a cysteine solution or to denatured egg albumin in solutions of urea, guanidine hydrochloride, or Duponol, these solutions no longer reduce ferricyanide (7). Nor do films of egg albumin reduce ferricyanide if mercuric chloride is present.

The number of SH groups found in films of egg albumin (by procedure II) is the same as in egg albumin denatured by solutions of urea, guanidine hydrochloride, or Duponol (7). Only in heat denatured egg albumin does the number of active SH groups seem to be different—0.5 to 0.6 per cent (8, 11, 6, 5, 1). This apparent difference is due to a curious oversight: In egg albumin denatured by urea, guanidine hydrochloride, Duponol, or rendered insoluble by surface forces, SH groups are estimated while the denaturing agent is still present (that is to say, estimations are made on albumin in a solution of the denaturing reagent or on albumin actually at

the surface), whereas in heat denatured egg albumin SH groups are estimated after the denaturing agent is removed (that is to say, on albumin

TABLE I
Reaction of Ferricyanide with Films of Egg Albumin

| Experi- ment No. | pH | Time of shaking | Albumin in solution | Precipi- tated albumin | Ferrocyanide formed | SH groups | SH groups in insoluble albumin |
|------------------------|------|--------------------|---------------------|------------------------------|---------------------|-------------------------|-----------------------------------------|
| | | <i>min.</i> | <i>mg.</i> | <i>mg.</i> | <i>mm</i> | <i>mg. cysteine</i> | <i>per cent cysteine</i> |
| I | 6.91 | 0 | 18.5 | 0 | 0 | 0 | — |
| | | 360 | 13.5 | | 0.000420 | | |
| | | | 13.6 | 5.0 | 0.000428 | 0.051 | 1.02 |
| | | | 13.5 | | | | |
| | | | Av. 13.53 | | Av. 0.000424 | | |
| | | 430 | 12.55 | | 0.000497 | | |
| | | | 12.30 | 6.0 | 0.000460 | 0.058 | 0.97 |
| | | | 12.75 | | 0.000497 | | |
| | | | Av. 12.53 | | Av. 0.000485 | | |
| | | 510 | 11.6 | | 0.000557 | | |
| | | | 11.6 | 7.1 | 0.000557 | 0.066 | 0.93 |
| | | | 11.0 | | 0.000537 | | |
| | | | Av. 11.4 | | Av. 0.00055 | | |
| II | 6.91 | 0 | 18.5 | 0 | 0 | 0 | — |
| | | 370 | 12.6 | 5.9 | 0.000464 | 0.056 | 0.95 |
| | | 440 | 11.5 | 7.0 | 0.000540 | 0.065 | 0.93 |
| | | 510 | 11.25 | 7.25 | 0.000568 | 0.068 | 0.94 |
| III | 6.56 | 0 | 18.5 | 0 | 0 | 0 | — |
| | | 300 | 11.3 | 7.2 | 0.00047 | 0.056 | 0.78 |
| | | 375 | 10.05 | 8.45 | 0.00061 | 0.073 | 0.86 |
| | | 435 | 9.25 | 9.25 | 0.00063 | 0.076 | 0.82 |
| IV | 7.44 | 0 | 18.5 | 0 | 0 | 0 | — |
| | | 380 | 14.25 | 4.25 | 0.000332 | 0.040 | 0.94 |
| | | 510 | 13.55 | 4.95 | 0.000405 | 0.0485 | 0.98 |
| | | 630 | 12.55 | 5.95 | 0.000458 | 0.055 | 0.93 |

that has been allowed to cool off). If SH groups are estimated in heat denatured egg albumin while the albumin is being heated, precisely the same number of SH groups is found as when estimations are made in the presence of urea, guanidine hydrochloride, Duponol, or surface forces.

In denaturation by heat, if the process is stopped while some egg albumin

still remains in solution, it is found that SH groups appear only in the albumin rendered insoluble, there being none in the albumin that was heated but not yet rendered insoluble. In denaturation by urea SH groups appear only in the fraction of protein with altered solubility (7). When films of insoluble egg albumin are constantly being formed, the fraction of albumin that still is soluble does not reduce ferricyanide. In thermal and urea

TABLE II

Reaction of Ferricyanide with Films of Egg Albumin after They Have Been Removed from the Surface. (Before Adding Ferricyanide the Albumin Had Been Shaken for 25 Hours and 63 Per Cent of It Had Been Coagulated)

Temperature 0–1°C.; pH 6.9

| Time of reaction with ferricyanide | Soluble albumin | Insoluble albumin | Ferrocyanide formed | SH groups | SH groups in insoluble albumin |
|------------------------------------|-----------------|-------------------|---------------------|--------------|--------------------------------|
| hrs. | mg. | mg. | mM | mg. cysteine | per cent cysteine |
| 6½ | 6.85 | 11.65 | 0.000342 | 0.41 | 0.35 |
| 20 | 5.5 | 13.0 | 0.000572 | 0.687 | 0.53 |
| 42* | 4.45 | 14.05 | 0.000745 | 0.895 | 0.64 |

* Total SH in albumin at this time, obtained by heating to 85° for 10 minutes.

0.00142 mM ferrocyanide was obtained. This is equivalent to 1.7 mg. cysteine or 0.92 per cent.

TABLE III

Reaction of Ferricyanide with Soluble Heat Denatured Egg Albumin

Temperature 0–1°C.; pH 6.9

| Time of reaction with ferricyanide | Ferrocyanide formed | SH groups | SH groups in albumin |
|------------------------------------|---------------------|--------------|----------------------|
| hrs. | mM | mg. cysteine | per cent cysteine |
| 1 | 0.00064 | 0.77 | 0.415 |
| 5 | 0.000828 | 0.993 | 0.54 |
| 6 | 0.000865 | 1.04 | 0.56 |
| 8 | 0.00087 | 1.045 | 0.565 |

denaturation and in the process of film formation, appearance of SH groups and alteration in solubility are integral parts of the same process.

When ferricyanide reacts with films of egg albumin after they have been removed from the surface (procedure I, Table II) the number of SH groups (0.6 per cent) found is the same as in egg albumin denatured by heat and then allowed to cool before SH groups are estimated (Table III). In both cases there is no sharp end-point to the reaction; it appears to continue almost indefinitely, although after some time at a much diminished rate. If at this time ferricyanide is washed away and the protein is tested with nitroprusside, no color reaction is obtained. Still the protein continues

to reduce ferricyanide, although very slowly indeed—as if SH groups were still present in the protein, but not fully accessible to ferricyanide. And there still are SH groups in the protein, for if the nitroprusside test is carried out in the presence of guanidine hydrochloride an intense color is obtained. The SH groups have not been lost by oxidation. The unreactive (or very sluggishly reactive) SH groups of films of egg albumin removed from the surface and the SH groups of heat denatured egg albumin, allowed to cool off, reduce ferricyanide rapidly if the ferricyanide-protein mixtures are heated to 85°. When egg albumin is denatured by a concentrated urea solution and the urea is subsequently diluted, the SH groups of the dissolved albumin no longer reduce ferricyanide rapidly (as they do in concentrated urea, the reaction being complete in less than 1 minute), but in the same sluggish and incomplete manner as in albumin denatured by heat and then cooled.¹ When the albumin in such a dilute urea solution is heated or when urea is added to the solution the SH groups immediately reduce ferricyanide.

The properties of egg albumin denatured by heat and urea that have just been briefly noted will be more completely described in another paper. They have been referred to in this paper because they show a close resemblance between surface films of egg albumin that have been removed from the surface, egg albumin denatured by heat and then allowed to cool, and egg albumin denatured by concentrated urea solution and the urea then diluted. There is also a close resemblance between the properties of films of egg albumin while they still are at the surface, albumin denatured by heat and kept heated, and albumin in solutions of urea, guanidine hydrochloride, or Duponol sufficiently concentrated to cause denaturation. In the many different ways of denaturing egg albumin the two most characteristic changes in the protein are the loss of solubility at the isoelectric point and the appearance of SH groups. These two changes also take place when a film of egg albumin is formed. In a film precisely the same number of SH groups appear as when egg albumin is denatured; and when the films are withdrawn from the surface, and allowed to clump together, the same change in SH groups takes place as when the denaturing agent is withdrawn from denatured egg albumin.

It is clear that the film at the surface of an egg albumin solution should

¹ When SH groups of denatured egg albumin disappear no native egg albumin is formed. All of the protein is still insoluble in water at the isoelectric point. That the amount of ferricyanide reduced by heat denatured egg albumin is increased by increasing the ferricyanide concentration or the time of reaction has been noted by Anson (1). The observations reported in the present paper were made before the publication of his paper.

be considered to be denatured. Apparently the same fundamental change takes place when a film of egg albumin is formed as when albumin is modified by heat, urea, and other agents. This would explain why the properties of a film of protein are not changed by heating to a temperature well above the temperature of denaturation of the same protein in solution (3); the change wrought in dissolved albumin by heat had already taken place when the film was formed.² The change in configuration of the egg albumin molecule that occurs when a film forms can in a general way be clearly described; the film, as has been said, is only 8 A.u. thick whereas the molecule in solution is an ellipsoid with a major axis of 91 A.u. and a minor axis of 32 A.u. This change in configuration would explain why SH groups appear in egg albumin. Groups in the interior of the protein molecule become exposed in a film and are thus able to take part in the reaction from which they had previously been shielded by the peripheral groups of the native protein molecule. The egg albumin molecule unfolds when a film is formed.³ Unfolding, and uncovering of the interior of a molecule, may also be supposed to occur whenever the SH groups of egg albumin give a reaction with nitroprusside and reduce ferricyanide—as they do when egg albumin is denatured by every agent that has so far been investigated. In another paper direct evidence will be given for the unfolding of egg albumin in concentrated solutions of urea.

Many SH groups in the films of egg albumin no longer reduce ferricyanide when the films are clumped together. These groups have not been oxidized. They simply are inaccessible to the ferricyanide. The albumin at this time is in the form of a suspension and (although the suspension is finely

² Dognon and Piffault who made the observation referred to (that a film of serum albumin heated to 70° is not changed) concluded that a thin film of albumin is not denatured by heat.

³ When the egg albumin molecule unfolds SH groups could conceivably react with ferricyanide for a reason quite different from the one that has just been given. If two SH groups must react simultaneously with ferricyanide to give one S—S group, it must be supposed that the two SH groups are placed close together in the protein molecule. It may be that in the native egg albumin molecule SH groups are so far separated from each other that it is impossible for two of them to react with ferricyanide to yield a S—S group. Unfolding would make possible the reaction with ferricyanide by bringing SH groups close together. This explanation is untenable because the SH groups of native egg albumin do not react with iodoacetate, and in this reaction SH groups do not react in pairs.

It has also been supposed that SH groups do not exist as such in the native protein molecule, that during denaturation they are formed from S—S groups. This theory will be considered in another paper.

divided) this may seem to be a sufficient reason for the inaccessibility of some SH groups to ferricyanide. Even in a *solution* of egg albumin, however, SH groups may become inaccessible to ferricyanide. This happens in a concentrated urea solution when the urea is diluted. And when heat denatured albumin is cooled its SH groups become inaccessible whether the albumin is dissolved or precipitated. An explanation for the disappearance of SH groups will be offered in another paper, when the phenomenon itself will be more completely described.

SUMMARY

1. The same number of SH groups reduces ferricyanide in surface films of egg albumin as in albumin denatured by urea, guanidine hydrochloride, Duponol, or heat, provided the ferricyanide reacts with films while they still are at the surface and with the denatured proteins while the denaturing agent (urea, heat, etc.) is present.

2. The SH groups of a suspension of egg albumin made by clumping together many surface films react with ferricyanide in the same sluggish and incomplete manner as do the groups in egg albumin denatured by concentrated urea when the urea is diluted or in albumin denatured by heat when the solution is allowed to cool off.

3. The known change in configuration of the egg albumin molecule when it forms part of a surface film explains why SH groups in the film react with ferricyanide whereas those in native egg albumin do not. In the native egg albumin molecule groups in the interior are inaccessible to certain reagents. A film is so thin that there are no inaccessible groups.

4. Because of the marked resemblance in the properties of egg albumin in surface films and of egg albumin after denaturation by the recognized denaturing agents, it may be supposed that the same fundamental change takes place in denaturation as in film formation—indeed, that film formation is one of the numerous examples of denaturation. This would mean that in general the SH groups of denatured egg albumin reduce ferricyanide and react with certain other reagents because they are no longer inaccessible to these reagents.

EXPERIMENTAL

The reagents used have been described in a previous paper (7). All experiments, unless otherwise noted, were done at 0 to 1°.

Procedure I.—In each of twenty-four 60 cc. glass stoppered pyrex bottles are placed 0.20 cc. of egg albumin solution (containing 18.48 mg. of egg albumin), 0.10 cc. of a 1 M pH 6.9 KH_2PO_4 - K_2HPO_4 buffer, and 9.2 cc. of water. The bottles are placed in a shaker making 240 strokes per minute. The shaker is placed in a cold room kept at a

fraction of a degree above zero. Even after shaking for more than 24 hours only two-thirds of the egg albumin is coagulated. Shaking is done in the cold to reduce to a minimum oxidation by the air in the bottles. (Actually little or no oxidation takes place. After shaking in the absence of ferricyanide for 25 hours and then with ferricyanide added for 45 hours the mixture was heated to 85° for 10 minutes. The quantity of ferrocyanide formed was the same as in a mixture heated at once, without any previous shaking.) After shaking for 25 hours, 0.10 cc. of 0.2 M ferricyanide is added to half of the bottles and all the bottles are replaced in the shaking machine. At various intervals of time four bottles (two with and two without ferricyanide) are removed from the shaker. In the mixtures containing ferricyanide the protein is precipitated by adding 0.3 cc. of 1 N H₂SO₄ and 0.1 cc. of a 10 per cent sodium tungstate solution. The protein is filtered off and the quantity of ferrocyanide is estimated, as previously described. To the albumin mixtures not containing ferricyanide 0.4 cc. of water is added and the suspension is then filtered. The protein content of the filtrate is estimated by the Kjeldahl method. From this the quantity of protein coagulated is reckoned by difference.

Comparison with Reduction of Ferricyanide by Heat Denatured Egg Albumin.—To 2 cc. of egg albumin solution are added 15.8 cc. of water and 2.2 cc. of 0.06 N HCl. The solution is heated at 85° for 10 minutes and is then cooled in an ice mixture. To 2 cc. of this solution (containing 18.48 mg. egg albumin) are added 7.4 cc. of water, 0.1 cc. of phosphate buffer, and 0.1 cc. of 0.2 M ferricyanide. These solutions of albumin and ferricyanide are shaken (although they are perfectly clear solutions) in the cold room. After various intervals of time to each sample are added 0.3 cc. of H₂SO₄ and 0.1 cc. of sodium tungstate. Ferrocyanide is then estimated in the protein-free filtrate.

Procedure II.—Ferricyanide is present in one-half of the bottles from the beginning of the shaking experiment. Various 1 M K₂HPO₄-KH₂PO₄ buffers are used—at pHs 6.56, 6.91, 7.44. In one experiment, with pH 6.91 buffer, 0.1 cc. of 0.1 M mercuric chloride is added to each sample of egg albumin at the same time that the ferricyanide is added. In this experiment the albumin is shaken for 10 hours.

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A NEW PIEZOELECTRIC MANOMETER TO RECORD INTRA-CARDIAC PRESSURES AND FOR THE SIMULTANEOUS RECORDING OF INTRACARDIAC ELECTROGRAMS*

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I

For the study of the effect of various agencies on the heart, it is frequently convenient to have simultaneous records of the electrical and mechanical manifestations of its activity. This can be accomplished in a number of ways; for instance, the classical form of the Frank-Wiggers manometer may be made to register pressure on part of the same film on which the electrocardiogram is being recorded. Although this method is capable of yielding accurate records, it is laborious and necessitates opening the chest of the animal and using artificial respiration. When two string galvanometers are at hand, a more convenient procedure is available. The pressure variations may be transformed into electrical changes that can be recorded with one of the instruments. In recent years the piezoelectric effect of Rochelle salt crystals has been utilized extensively for recording sounds and other vibrations, and it seemed probable that this method might also be employed to record pressure changes.

The apparatus (Fig. 1) which we devised for accomplishing this consists of a rugged housing (Fig. 1B 1), inside which a large slab of crystal (Fig. 1A c, and B 3) is secured at one end. The free end of the crystal is in contact with a diaphragm (Fig. 1A b, and B 5) which closes the end of a long, narrow cannula (Fig. 1A a and B 7), filled with saline solution. If certain limits are not exceeded, such a crystal can be made to produce charges which are accurately proportional to the pressure distorting it.

The problem of constructing an appropriate recording system involves two other considerations. First, the crystal must be connected to the string galvanometer in such a way that the static charges which develop under pressure are not dissipated too rapidly. This is accomplished by using a vacuum tube with a low grid current to couple the crystal to the galvanometer. Since the voltages developed by the crystal are relatively high, no amplification of the effect is needed, and a very simple circuit may be used.

* A preliminary report of these observations appeared in the Journal of Clinical Investigation 17: 510, 1938.

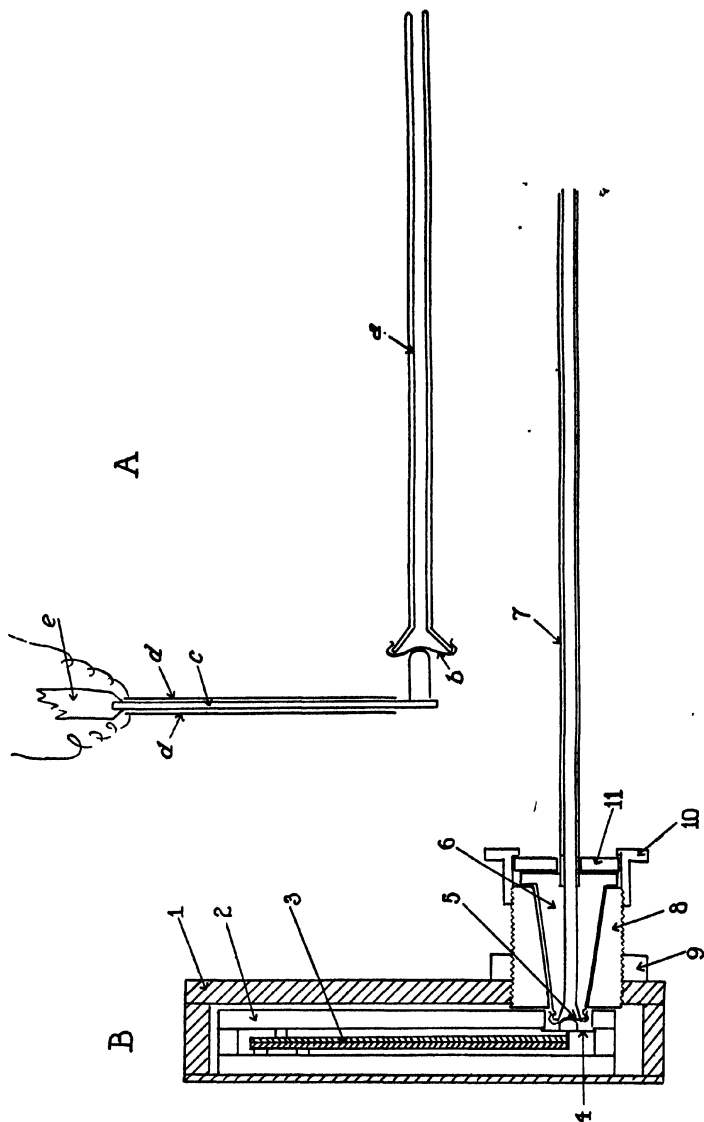


FIG. 1. Piezoelectric manometer. A, Schematic drawing to illustrate the principle of operation. *a*, Cannula filled with salt solution; *b*, Distensible membrane which covers the end of the cannula and impinges upon a button connected to the free end of the crystal, *c*; *c*, slab of Rochelle salt crystal, held rigidly at one end by support *e*, and covered on both surfaces by plates of tin foil, *d*. When the open end of *a* is in-

The coupling of the crystal to the source of pressure is equally important. In this case the problem is to construct a system with a sufficiently high-frequency response. For this purpose a long cannula filled with salt solution is used. The end in contact with the crystal is closed with a very tightly drawn rubber diaphragm; the end which is inserted into the heart is left open. To prevent clotting, heparin is added to the salt solution with which the cannula is filled. The cannula is of small enough bore and sufficient length to be inserted into the heart by way of the jugular vein or the carotid artery. The cannula with its diaphragm is constructed as a single unit (Fig. 1B 5, 6, and 7) which is readily detachable from the crystal housing. It is filled with salt solution by means of a capillary pipette, and freed of air bubbles. Since the bore is so small that there is no danger of spilling the fluid, it is as easily manipulated as an empty cannula. The crystal housing, which is relatively large, need not be attached until the cannula is in the heart. A number of cannulae of different sizes are prepared at the beginning of the experiment, so that the appropriate size may be selected after the vessels are exposed.

Hamilton, Brewer, and Brotman¹ have discussed at length the frequencies attainable through narrow cannulae, and they probably adequately demonstrated their contention, but their work has not escaped criticism. It will not be redundant, therefore, to discuss from a different point of view the factors upon which the frequency responses of cannulae depend.

The cannula of length l (Fig. 2) is closed at one end by a distensible membrane (M), and connected to a chamber (H) in which the pressure varies. If the pressure in H is increased from P_1 to P_2 , the membrane will assume the form M' , and, consequently, an amount of fluid designated by s must pass through the tube. The speed with which this transfer can take place is the limiting factor in the frequency response of the cannula.

The principal forces which tend to retard the flow of fluid through the tube are

serted into a chamber of the heart, the pressure changes which occur within it cause a distention of the membrane, b , which, in turn, bends the slab of crystal, c . This deformation of the crystal causes its faces, which are covered by the tin foil, to assume opposite electric charges. If certain limits are not exceeded, these changes are proportional to the amount of deformation of the crystal, and, consequently, to the changes in pressure. *B*, Plan showing the actual construction of the manometer. 1, Rigid duraluminum outer case; 2, fiber inner case; 3, crystal; 4, diaphragm closing fiber case to protect crystal from moisture; 5, very tightly drawn rubber diaphragm, closing end of cannula; 6, the brass cone which forms the end of the cannula makes possible a very rigid connection between the cannula and the case of the crystal; 7, the cannula is made of silver and insulated with baked-on lacquer, save for the tip; 8, the receptacle for the conical end of the cannula is threaded, so that the contact between the diaphragm closing the end of the cannula and the button connected to the free end of the crystal can be adjusted; 9, a lock nut to hold the receptacle rigidly once the correct position is found; 10, a threaded collar with an opening large enough so that it can be slipped over the conical end of the cannula after it has been inserted into the animal's heart; 11, a slotted disk which can be slipped around the cannula after it is in place to engage the threaded collar.

inertia and friction. When small cannulae are used, the inertia of the fluid can be neglected, because it decreases with the bore. According to King's formula, the retarding effect of friction is $h_f = K \frac{l}{d^{1.25}} V^{n*}$. K is a constant which depends upon the roughness of the tube; l and d are the length and diameter of the tube, and V is the mean velocity in centimeters per second. If this formula is expressed in terms of s , the amount of fluid passing through it in time t (the time taken for the pressure to change from P_1 to P_2), it becomes $h_f = 4K \frac{ls^2}{td^{5.25}}$.

From the last equation it is apparent that an increase in length of the cannula (l) increases the resisting force, but that a decrease in diameter (d) increases it much more because this factor appears as the fifth power. Both of these effects can be counteracted, however, by a sufficiently great decrease in s (the distensibility of the membrane), which appears as the second power. Although it is clear from the equation what factors are involved, the precise extent to which a decrease in diameter can be compensated for by a decrease in the distensibility of the diaphragm cannot be predicted from it, but must be ascertained experimentally. The experiments of Hamilton, et al.,¹ have established the fact that cannulae much smaller than it has been necessary to use to enter the hearts of dogs and cats by way of the vessels in the neck can be employed without distorting the curves when the distensible diaphragm is a thin silver plate. The displacement (s) of such a diaphragm is very small. Since even less movement of the diaphragm is required to operate the crystal than Hamilton requires for his optical method of recording, his experiments are applicable to the cannulae which are used with the piezoelectric recorder.

A convenient record of electrical activity to pair with the pressure curve is one which emphasizes the effect of the chamber that is being explored. This is particularly desirable in the case of the auricle, for, in ordinary electrocardiograms, the electrical effects of its activity are so small that no detailed analysis of them is possible. Since Wilson and his collaborators^{2,3} have shown that, when one electrode is very close to the heart, it is affected predominantly by the muscle closest to it, this result is easily accomplished by using the tip of the cannula as the exploring electrode. To this end, the cannula is made of silver and insulated, save for the tip, with baked-on enamel. The exposed tip is rendered nonpolarizable by giving it a coat of silver chloride. A silver plate, inserted beneath the skin of the animal's hind leg, serves as the indifferent electrode. An added advantage of using the tip of the cannula as the exploring electrode is that any impingement of the heart wall that might occlude or partially occlude its orifice is at once betrayed by the appearance of a monophasic response in the electrical record (Fig. 3II and III).

The complete assemblies, including cannulae and crystals, have inherent frequencies in excess of 100 per second, as indicated by the usual tests, but since the ability of this instrument to record the slow components of the curves must also be checked, a direct comparison was made with the classical form of the Frank-Wiggers manometer by inserting the cannula of the crystal

* n is usually 2, but may vary from 1.75 to 2.08.

recorder into the femoral artery of a dog, and that of a Frank-Wiggers manometer into a branch close enough to the main stem so that the tip protruded into it. In this way the mouths of the two cannulae were brought very close together. Practically identical curves were obtained from the two instruments (Fig. 3I). The cannula used on the crystal manometer in this instance was the same length, but smaller in diameter, than those used to enter the heart.

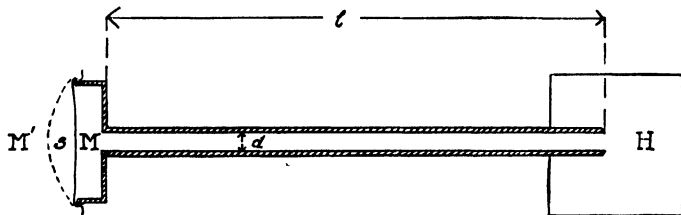


FIG. 2

II

As the cannula is passed down the jugular vein of an animal, the pressure curves gradually increase in size until a typical intra-auricular pressure curve is obtained (Fig. 3IV and V). These curves are similar in contour to those of previous observers.¹ They nearly always show some rapid vibrations at the time of the occurrence of the heart sounds, particularly the first. When the cannula is pushed into the immediate vicinity of the tricuspid valve, these vibrations become marked (Fig. 3VI). When the cannula passes through the valve into the ventricle, the character of the curve changes abruptly into that of a typical intraventricular pressure curve which usually shows no trace of the vibrations of the first heart sound (Fig. 3VII, VIII, and IX).

When the cannula is passed down the carotid artery, the carotid pulse which is recorded usually shows some trace of the second heart sound. When the cannula is close to the aortic valve, these vibrations are of considerable amplitude (Fig. 3X). After the ventricle is entered, the character of the curve abruptly changes to the intraventricular type (Fig. 3XI).

In the electrogram obtained from the interior of the right auricle the auricular complex is large, and both parts of it can be made out, i.e., the rapid accession process (A_1 , Fig. 3V) and the regression or T deflection (A_2 , Fig. 3V). The latter is usually combined with the ventricular part of the electrogram, but in many instances it can be seen quite clearly. A_1 is usually diphasic, and the first phase is downward. When the cannula is near the junction of the great veins with the auricle, this downward phase is small (Fig. 3V); near the auriculoventricular junction it is larger; and in the upper part of the ventricle

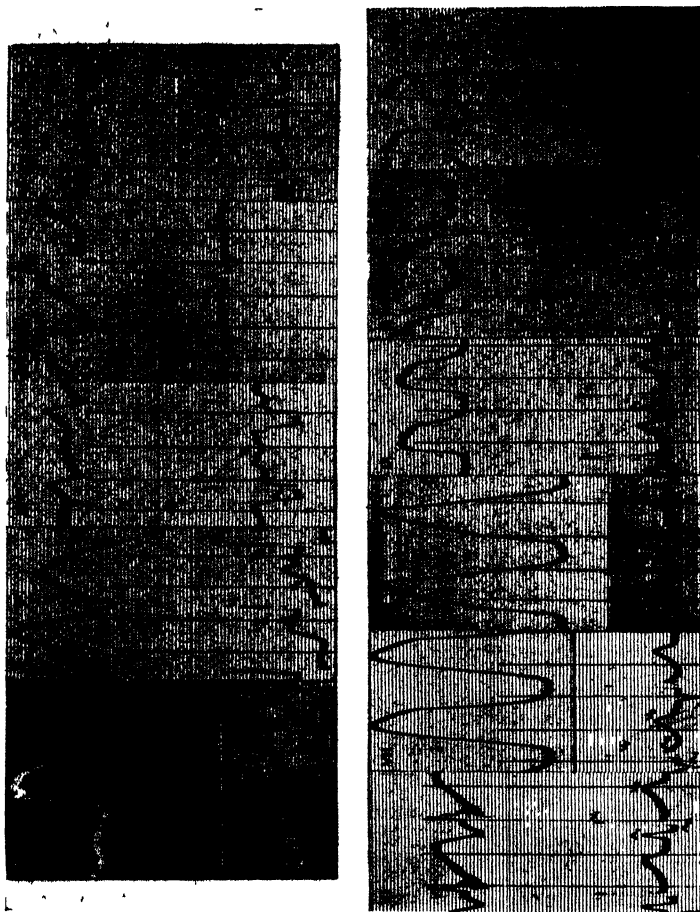


FIG. 3. *I*, A comparison of curves obtained with piezoelectric and Frank-Wiggers manometers. Top (white) curve, femoral pulse of a dog as recorded by the Frank-Wiggers manometer; middle (black) curve, femoral pulse recorded by the piezoelectric manometer. The bottom curve is a simultaneously recorded electrocardiogram. *II*, Curves which exhibit the effect of inserting the cannula too far into the right ventricle of a cat, so that its tip came in contact with the wall. The pressure curve is deformed (cf. *VII*, *VIII*, and *IX*), and the ventricular electrogram tends to be of a monophasic type (the take-off of the T wave is below the isoelectric line). *III*, Curves which exhibit the effect of inserting the cannula too far into the right auricle of a cat, so that its tip came in contact with the auricular wall. The pressure curve is deformed (cf. *IV* and *I*), and the auricular electrogram tends to be of the monophasic type (*A*₂). *II'*, Typical records from the right auricle of a cat obtained

(Fig. 3VII) it (the downward phase) alone remains. The auricular regression deflection, as a rule, consists of a small, rounded, downward or upward deflection (A_2 , Fig. 3V and VII) between V_1 (QRS) and V_2 (T). In some of the earlier experiments no insulating lacquer was used on the outside of the cannula; under these circumstances A_1 consisted of a single upward phase (Fig. 3IV) which did not change in shape when the cannula was pushed into the ventricle (Fig. 3VIII).

III

Usually, electrograms from the interior of the two ventricles are similar; they consist of a single upward (negative) deflection (V_1) of large amplitude, followed by an upward, downward, or nearly isoelectric T wave (V_2) (Fig. 3IX and XI). Occasionally, from the right ventricle, curves are obtained in which the initial component is diphasic; the initial (V_1) upward deflection is followed by a downward (positive) deflection (Fig. 3VII and VIII). The ventricular complexes obtained when the cannula is still in the auricular cavity resemble those from the ventricle, except that they are not so large.

with an uninsulated cannula. The pressure curve (upper) shows an initial spike, a , caused by contraction of the auricle, followed, after a brief interval, by a sharp fall, b , and a subsequent, gradual rise, c , which are caused by ventricular activity. The electrogram (lower curve) resembles an electrocardiogram. Auricular activity is registered by an upward spike (A_1), followed by the two components of the ventricular complex, namely, V_1 (= QRS) and V_2 (= T). V , Records from the right auricle of a cat obtained with a cannula which was insulated, save for the tip. The pressure curve resembles IV, except that there is an additional positive phase, d , caused by auricular filling. In the electrogram (lower curve) the auricular response (A_1) is diphasic and of much greater voltage than IV. It resembles the intrinsic responses which are obtained when an electrode is placed in direct contact with the exterior of the auricle. The regression process of the auricle (A_2) is superimposed upon V_1 , which is the initial ventricular response. VI, when curves are recorded with an uninsulated cannula in close contact with the tricuspid valve of a cat, the first heart sound appears in the pressure curve. VII, Curves recorded with an insulated cannula in the right ventricle of a cat; A_1 , the auricular accession process, is downward, as is the intrinsic deflection from the exterior of the heart at the auriculoventricular junction. A_2 , The auricular regression process, is upward and continued with V_1 , which is diphasic; the second component is large and downward. VIII, Curves obtained with an uninsulated cannula in the right ventricle of a cat. V_1 is diphasic. IX, Curves from the right ventricle of a cat. V_1 is representative of the more usual monophasic type. X, Curves obtained with an insulated cannula in the aorta of a dog, near the aortic valve. The vibrations of the second heart sound can be seen in the pressure curve. A_1 resembles intrinsic deflections from the region of the sinus node. XI, Curves obtained when an insulated cannula is passed into the left ventricle of a dog. V_1 is monophasic.

If the crystal in the manometer is deflected by subjecting the cannula suddenly to a change of pressure, the crystal faces become charged, but these charges will gradually leak away, even when the pressure is maintained constant. Since the time required for these charges to dissipate is ten seconds or more, the rapidly changing pressures which accompany cardiac contraction are recorded accurately, but constant pressures are not recorded at all. Consequently, only pulse pressures can be measured with this instrument. For many purposes, however, this is sufficient, particularly if information is desired about the force of cardiac contraction rather than changes in the peripheral resistance. Since the crystal, when deflected, behaves like a condenser discharging through a high resistance, the lower the voltage, the less the rate of discharge, so that, if a relatively small voltage is produced by deflecting the crystal only slightly, the accuracy of the record is increased. Since only a few millivolts are required to give records of good amplitude, one may use very stiff diaphragms which not only improve the performance of the crystal, but increase the frequency response of the cannulae.

The pressure curves obtained from the three chambers of the heart which can be explored with this type of manometer are similar to curves obtained by other appropriate means.⁴ The value of the new instrument depends on its convenience and on the fact that the animals can be left intact and their circulation not interfered with. It has the advantage over the method of Hamilton, et al.,¹ which may also be used without opening the animal's thorax if a needle is thrust through the chest wall into the heart, that there is less uncertainty as to the position of the cannula, and that the animal need not be near the recording camera. The animal can be studied in an adequate light without interfering with photographic recording.

Only one type of pressure curve requires special comment, namely, that obtained in the immediate vicinity of the heart valves. These records may show sound-like vibrations which are coincident with valve closure (Fig. 3VI and X). These vibrations may be artifacts caused by impingement of the valves on the end of the cannula, or may be produced by closure of the valves (the valvular component of the heart sounds). Against their being artifacts is the fact that plucking (setting into vibration) the end of the cannula produced only very small vibrations in the record. It was, of course, necessary to construct the manometer so that sidewise tugging on the cannula would not distort the records with artifacts caused by motion of the heart. Obviously, after the cannula has entered the ventricle, the valve leaflets must still be impinging upon the cannula, but, nevertheless, the vibrations disappear. Brewer, Hamilton, and Brotman⁶ have shown that very abrupt changes in pressure may set the recording system into vibration if its inherent frequency is not sufficiently high, but pressure changes quite as abrupt as those which occur at these points in the curves are not always associated with sound-like

oscillations, notably the sudden rise in pressure during the isometric contraction phase. The conception of Brewer, Hamilton, and Brotman that practically all cardiovascular sounds are in reality caused by sudden changes in pressure which set the recording system or the eardrum into vibration is probably too sweeping. There can be no doubt that the heart sounds, for instance, are really sounds (repeated oscillations) at the anterior chest wall, for microphones capable of responding to frequencies as high as 30,000 cycles per second always record them as sounds consisting of many oscillations. Sudden changes of pressure occur when the myocardium contracts or the valves close, and these changes, when they are applied to adjacent inert tissues, will inevitably cause them to vibrate. Therefore, the first and second heart sounds cannot be considered as instrumental vibrations unless all of the tissue between the source of shock and the surface of the body is regarded as part of the recording instrument. Although the vibrations which are recorded from the immediate vicinity of the valves by means of this new manometer may be some sort of artifact, they may also reflect the vibrations, caused by closure of the valves, which are transmitted to the surrounding tissues as the valvular components of the heart sounds.

Electrograms from the interior of the heart have not been recorded frequently,⁶ for, although they have considerable theoretical interest, they have been of little practical value because of the difficulty in obtaining them. Since they are easily obtained with this new device and may prove to be useful records of the heart's electrical activity, they should be discussed briefly.

Wilson, Macleod, and Barker⁸ showed that electrograms taken with an exploring electrode in contact with the auricle show intrinsic deflections which are similar to those obtained from the ventricle, but they can be obtained only when the electrode is very close to the auricular surface, whereas intrinsic-like deflections can be obtained from the ventricle even through the chest wall.⁷ The electrograms from inside the auricle which were recorded in the course of this study resemble those taken from comparable positions on the outside. This similarity is to be expected, because an impulse which spreads radially bears the same relative position to any electrode which it passes, whether the latter be inside or outside the auricle, and because the conductivity of the blood is not very different from that of the tissues which surround the heart.

These electrograms from within the auricle are larger than those which Brown⁸ obtained with an electrode in the esophagus, but not quite as large as those obtained by the usual methods from the exterior surface. The auricular complexes of the curves obtained from the aorta near the aortic valve (Fig. 3X) resemble Brown's curves closely.

Although it is impossible to know accurately to what part of the auricular muscle the tip of the cannula is nearest, the change in the shape of the curves as the cannula is pushed through the auricle is what would be expected from the

work of Wilson, Macleod, and Barker.⁸ An early intrinsic deflection, with a small, preceding, downward deflection, is obtained when the cannula first enters the auricle by way of the superior vena cava and is near the sinus node (Fig. 3V, A_1). In the auriculoventricular region the intrinsic deflection is late, and, in the upper part of the ventricle, it disappears, and the complex consists of a downward deflection only (Fig. 3VII, A_1). Curves of an intermediary type (the upward and downward phases of which are more or less equal) are not usually obtained because the cannula passes through the auricle from the orifices of the great veins to the auriculoventricular region without coming in close contact with the mid-portion of the auricular wall.

Because it is too small, the P wave of the electrocardiogram has been of comparatively little use in studying the condition of the auricular myocardium. It has, for instance, been known for a long time that the P wave is only that part of the auricular activity which corresponds to the QRS group of the ventricular complex; the part corresponding to the T wave is usually too small to be seen.* In electrograms taken directly from the surface of the auricle, and in Brown's curves from suitable positions in the esophagus, the T portion of auricular activity can be readily identified. In normal curves from the interior of the auricle, this TA deflection (Hering), or auricular regression deflection (Macleod¹¹), usually falls in the QRS complex, and, consequently, is not easily recognized (Fig. 3V and VII, A_2). It may, however, as Brown has pointed out, deform the S-T segment; it may also account for the failure of the curve to return to the isoelectric line during the P-R interval. As will be shown in a subsequent paper,⁹ alterations in this process are often very easily seen, although, in its normal form, it is inconspicuous. That the auricular regression deflection is a delicate index of the effect of drugs and other agencies on the auricular myocardium has been shown by Cohn and Macleod,¹⁰ and Macleod.¹¹ It is, therefore, particularly desirable to be able to study this deflection in mammals.

Wilson, Johnston, and Hill,¹² in their study of myocardial infarction, described electrograms which were taken from the interior of the ventricles. The QRS of their curves consists of a single, tall, upward (negative) deflection. This is the type of curve which is to be expected if the impulse is conducted rapidly over the endocardial surface by means of the Purkinje network, and subsequently spreads outward through the ventricular wall at a slower rate. The curves which were obtained from the left ventricle in the course of the present study were always of this form. Those from the right ventricle were usually of the same type, but occasionally, both in dogs and cats, curves were encountered which showed a prominent downward (positive) deflection after the upstroke. The explanation of this deflection is not at once apparent; its

* Brown's paper⁸ contains a full account of the history of this deflection.

presence might seem to be inconsistent with the view of Wilson, Johnston, and Hill, but, by utilizing the same principles upon which their concept is based, and taking into consideration certain anatomic facts, one can account for it.

Wilson, Macleod, and Barker¹⁸ have explained how the active process can be likened to an expanding shell, so polarized that its outward surface is

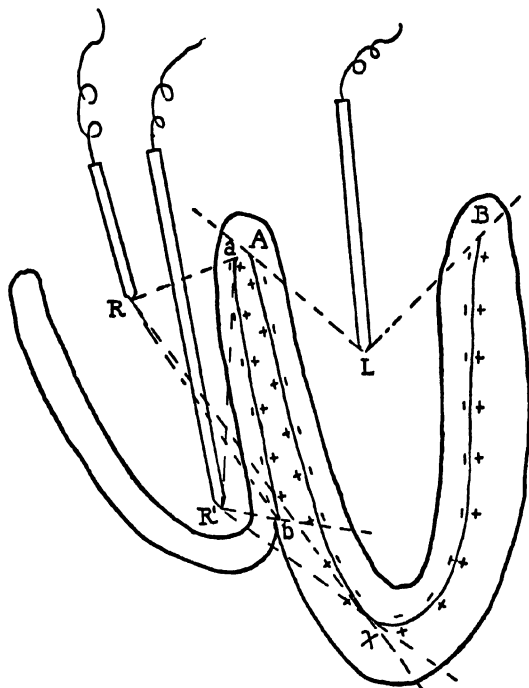


FIG. 4

positive and its inner surface is negative, spreading outward through the ventricular muscle. An electrode in the left ventricle (*L*, Fig. 4) is exposed only to the negative surface of this shell throughout the period of excitation. Although it is true that the septal portion of the right ventricular shell has its positive surface towards electrode *L*, the effect of this is always neutralized by the septal part of the left ventricular shell.

Electrode *R*, in the right ventricle, like *L* in the left, will be negative until its expanding shell reaches the outer surface of its lateral wall and disappears. Now only the septal portion remains. If the electrode is so placed (*R*) that this portion masks the positive surface of the left ventricular shell, it will

remain negative or become zero, but, if the electrode is at R' , this septal portion of the right ventricular shell is insufficient to do this, and the electrode will become positive during the later part of the period of excitation.

SUMMARY

1. A manometer which simultaneously records intracardiac pressure curves and intracardiac electrograms is described.
2. Criteria are presented for estimating the accuracy of the pressure curves obtained with this apparatus.
3. Electrograms obtained with the noninsulated tip of the manometer as it is pushed progressively farther through the right auricle, the A-V valve, and into the ventricle demonstrate changes in the action currents corresponding to these levels.
4. The form of the electrogram obtained from within the ventricle is likewise discussed.

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THE EFFECT OF ACETYLCHOLINE ON THE MAMMALIAN HEART

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Cohn and Macleod¹ have recently published a study of the effect of mecholyl and acetylcholine on the frog's heart. This study indicated the general mode of action of the drug, but, because choline derivatives are being used to a considerable extent clinically, particularly for the relief of attacks of paroxysmal tachycardia, and because the vagus mechanism of mammals is somewhat different from that of frogs, it seemed desirable to extend these studies to the mammalian heart. The effect of the drugs on the mechanical response of the heart could, furthermore, be more easily studied in the mammal because of its greater size.

The experiments were carried out on cats and dogs which were anesthetized with nembutal. The vagi were exposed and cut. Stimulating electrodes were attached to their peripheral ends. The cannula of the piezoelectric manometer, insulated except at its tip, which was described in a previous paper² was inserted into the heart by way of a jugular vein or carotid artery. The intracardiac pressure pulse was recorded, together with an electrogram derived from the tip of the cannula (exploring electrode) and a silver plate inserted under the skin of the left hind leg (indifferent electrode). Simultaneous records of the electrical and mechanical activity of the heart were obtained by this means without opening the animal's thorax. Records were obtained from the right auricle and the right ventricle by pushing the cannula through a jugular vein, and from the left ventricle by inserting it into a carotid artery. The drugs were administered intravenously. A dose of 1 to 2 mg. of acetylcholine (or about one-tenth that amount of mecholyl) was used for the average cat; dogs required 5 to 7 mg. of acetylcholine to obtain an equivalent effect.

II

Auricles

The earliest effect of stimulation of either vagus is slowing of the auricular rate. Accompanying, or immediately following, this, there is diminution in the force of auricular contraction (negative inotropic effect, Fig. 1 *II*, and *III*). At about the same time there are alterations in the auricular regression deflection (Fig. 1 *III*, *A*₂). After strong stimulation, marked shortening of this

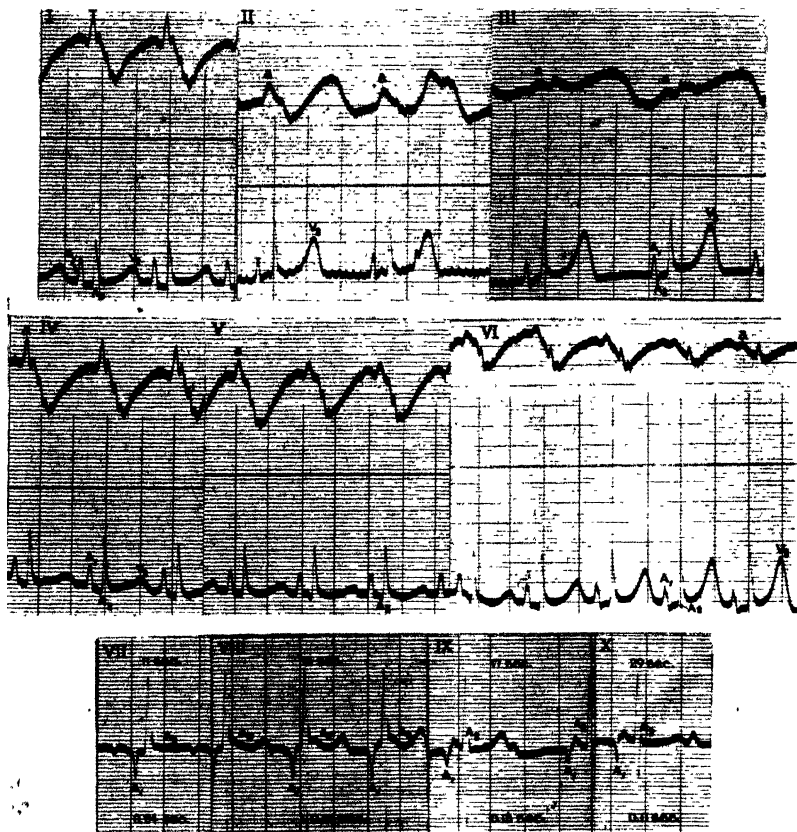


FIG. 1. The effect of vagus stimulation and acetylcholine on the intra-auricular pressure curve during auricular contraction. *a* is the component of the auricular pressure curve caused by auricular contraction. A_1 represents the accession process in the auricular electrogram (P wave). A_2 is the regression process in the auricular electrogram (Ta wave). V_2 is the regression process of the ventricular electrogram (T wave). *I*, *II*, and *III* were taken before, during, and just after left vagus stimulation. *II* and *III* show a negative inotropic effect in the auricles. They also exhibit small ripples caused by escape of the stimulating current. *IV* was taken before injection of acetylcholine; *V*, seven seconds after the injection of 2 mg. acetylcholine; and *VI*, about ten seconds after injection. *VII*, *VIII*, *IX*, and *X* are electrograms obtained with the electrode inserted just inside the ventricle of a cat. They show shortening of electrical systole and a decrease in voltage of the auricular complex. The figures above each curve give the time after an injection of 2 mg. of acetylcholine; those below, the duration of electrical systole (from the beginning of the accession process to the end of the regression process).

process occurs. The first observable effect, however, is a deformity of the segment which immediately follows the accession deflection (in the P-R region). It is difficult to be certain whether these changes precede, accompany, or follow the reduction in the force of contraction (the relationships differ in different experiments), but, in any case, they are nearly coincident with it. During the height of vagus action the amplitude of the whole of the auricular electrogram is reduced (Fig. 1 *III*). Prolonged stimulation occasionally initiates a brief paroxysm of auricular fibrillation.

Acetylcholine produces similar results (Fig. 1 *II'*, *V* and *VI*), except that the changes are of greater magnitude; that part of the intraauricular pressure curve which results from auricular contraction may entirely disappear (Fig. 2 *I*). The auricular regression deflection is greatly shortened (Fig. 1 *VII X*). (Since heart block nearly always occurs at this stage, the changes in this process are undistorted by any ventricular effects.) The reduction in size of the entire auricular electrogram is marked (Fig. 2 *I*). Auricular extrasystoles usually occur (Fig. 2 *I*, *I'*), and, if the dose has been large, one of these may initiate an attack of auricular fibrillation (Fig. 2 *I*, *I''*). This invariably starts as a series of rapid regular oscillations (state of rapid re-excitation) which sometimes are almost like those of alternating current (Fig. 2 *I* and *IV*, but, at other times, in each cycle, a more rapid and a slow component can be made out; these correspond to the accession and regression deflections. During the early stages of the attack ventricular contractions are infrequent, possibly because of the ineffectiveness of such small and rapid auricular stimuli, but more probably because of the coincident occurrence of auriculoventricular heart block. Then a stage usually occurs wherein the fibrillation waves are smaller and less regular (Fig. 2 *II*). As the effect of the drug wears off, the waves become coarser, until a state of impure flutter is reached; this finally gives way to normal rhythm (Fig. 2 *III VII*).

Recovery from a single injection is apparently complete, but after repeated injections the heart weakens and the animal may die.

A-I' Structures

As has been mentioned, when a sufficiently large dose is given, heart block, complete or partial, invariably occurs (Fig. 2 *I*).

Ventricles

The effects of vagus stimulation on the ventricles are similar in kind to those produced on the auricles, but much less in degree. For the first few beats after vagus stimulation is begun, the intraventricular pressure curve may increase in size as a result of the increased diastolic filling (Fig. 3 *II*) which is made possible by bradycardia. Subsequently, the beats may diminish (Fig. 3 *III*) in strength, but this diminution is never so great as in the case of

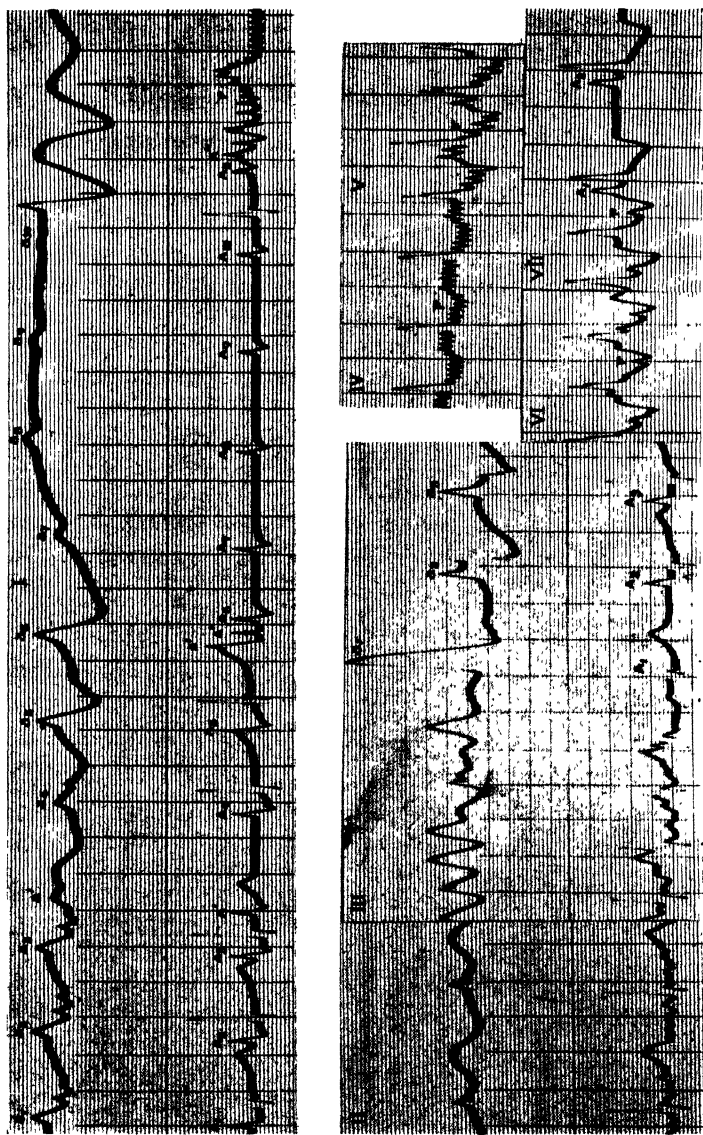


FIG. 2. The occurrence of auricular fibrillation after the administration, intravenously, of acetylcholine to cats. a_1 , a_2 , etc., result from the rise in pressure caused by regular auricular contraction; and a^1 , a rise in pressure caused by an auricular premature beat. A_1 , A_2 , etc., represent auricular electrograms, and F , waves of fibrillation. *I*, *II*, and *III* were taken from the same experiment. The tip of the

the auricles. The extent of the negative inotropic effect varies greatly in different preparations.

The ventricular electrogram also shows less dramatic changes than the auricular. Shortening of ventricular systole cannot be made out with certainty, but the terminal portion of the regression deflection (T wave) is definitely increased in height (Fig. 3 *III*). With prolonged stimulation there is possibly slight reduction in the voltage of the accession deflection (QRS). Ventricular fibrillation following vagus stimulation did not occur in any of the experiments.

Acetylcholine also exerts less effect upon these chambers than upon the auricles. An unmistakable reduction in the force of contraction usually occurs within a few cycles after the administration of the drug. This is accompanied by an increase in the height of the regression deflection (T wave, Fig. 3 *VI* and *VII*). There is often a definite reduction in the amplitude of the accession deflection (QRS). Ventricular extrasystoles occur occasionally, as does ventricular fibrillation. The latter usually persists long enough to cause death.

III

A reduction in the intracardiac pressure pulse must indicate a negative inotropic effect unless the heart is inadequately supplied with blood or is contracting against reduced resistance. In the case of the auricles, after the administration of acetylcholine, it is inconceivable that they are inadequately supplied with blood, for both sinus bradycardia and heart block are present; the former allows adequate time for filling, and the latter retards the removal of blood. These conditions also suggest that the auricles must contract against increased, rather than reduced, resistance. If the heart rate is slow enough so that the ventricles have been passively filled with blood before the auricles contract, the resistance against which the auricles work is the venous pressure, and this is increased. It is, therefore, certain that the reduced amplitude of the auricular pressure curve indicates a genuine, negative, inotropic effect of the drug.

cannula lay in the auricle, just beyond the entrance of the superior vena cava. *I* starts nine seconds after the injection of 2.5 mg. of acetylcholine, and shows a decrease in the vigor of auricular contraction (a_7 , a_8 , a_9 , and a_{10}), great shortening of the auricular regression process ($.14$ -. 1_{10}), heart block following $.1_5$, and auricular fibrillation following $.1''$. *II* starts one minute after injection. *III* starts two minutes after injection. It shows the cessation of auricular fibrillation; the first normal auricular beat, $.1_1$, is superimposed on a ventricular complex. It consequently produces a large rise in pressure, a_1 . $.1_2$ and $.1_3$ are normal in contour, and give rise to normal pressure changes (a_2 and a_3) similar to $.1_1$ and a_1 in *I*. *IV*, *I*, *VI*, and *VII*, from the same experiment, occur one, two, two and one-half, and three minutes after the injection of 3 mg. of acetylcholine. The abnormal rhythm is more in the nature of flutter (the waves remained regular); the decrease in rate can be easily followed, therefore, as the effect of the drug wears off. In *VII*, normal rhythm is resumed.

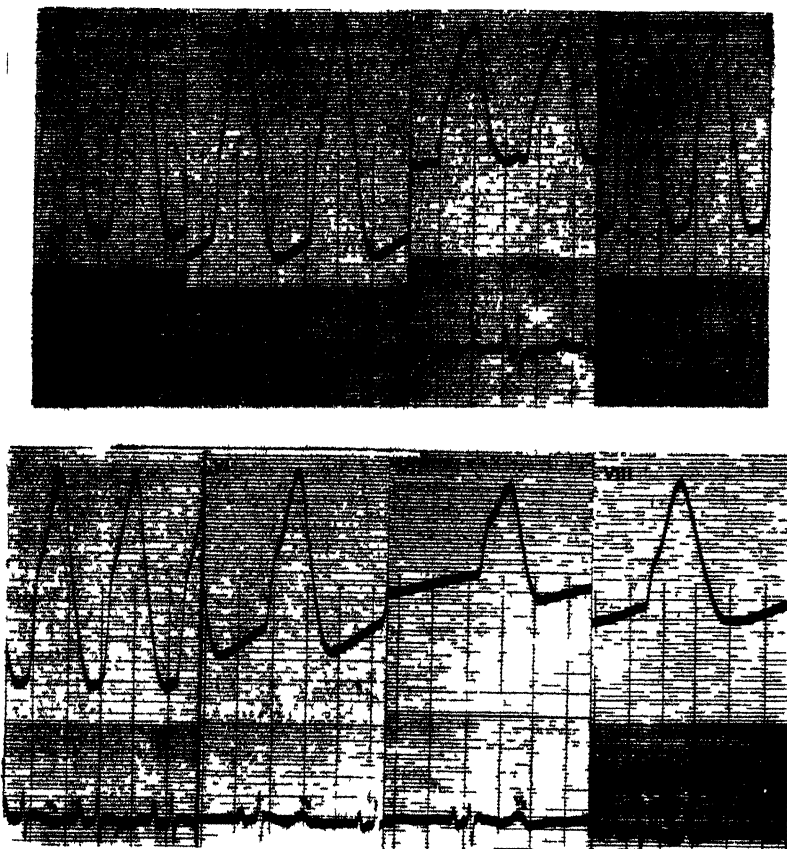


FIG. 3. The effect of vagus stimulation and of mechohyl on the ventricles of cats. *I* represents the regression deflection (I wave) of the ventricular electrogram. All of the curves are from the same experiment. *I* was taken before vagus stimulation. *II*, one second after the start of faradic stimulation of the peripheral end of the left vagus, it shows a transient increase in the amplitude of the ventricular pressure pulse (the stimulus artifact can be seen). *III*, fifteen seconds after the beginning of vagus stimulation and five seconds after its cessation, it shows a decrease in the ventricular pressure pulse and an increase in the height of the I waves. *IV*, fifty seconds after the cessation of stimulation, it shows the return of the mechanism to normal. *V*, *VI*, *VII*, and *VIII* occur before, five seconds, forty five seconds, and one minute, respectively, after the injection of 0.15 mg. of mechohyl. The diminution in amplitude of the pressure pulse and the increase in height of the T wave are shown. In *VIII*, heart block is present. The electrogram has returned almost to its normal form, but the pressure pulse is still smaller than the control.

The reduction in the ventricular pressure curve can be less certainly ascribed to a depressant effect on the myocardium, for, although the slow ventricular rate assures adequate filling of the ventricles, a reduction in the diastolic blood pressure could reduce the resistance against which it contracts sufficiently to account for part or all of its reduction in vigor. Following stimulation of the peripheral end of the vagus, it is not likely that much lowering in blood pressure occurs, however. The change in the shape of the electrogram indicates, furthermore, that some myocardial change has occurred. It is probable, therefore, that both vagus stimulation and the administration of acetylcholine cause some reduction in the force of ventricular contraction. Drury³ failed to observe a negative inotropic effect on the ventricles as a result of vagus stimulation with the Cushny myocardiograph, but the method of investigation used in this study is probably more sensitive and might show slight changes which the older method could not detect. In any case, the ventricular effects are of a different (much smaller) order of magnitude from those which occur in the auricles.

The alterations in the electrograms indicate, accordingly, an effect on the whole heart, but predominantly on the auricles. The shortening of electrical systole in the auricles is strikingly similar to that observed by Cohn and Macleod¹ in the frog's heart. Although estimations of the refractory period were not made in this series, it is well known from the work of Lewis, Drury, and Bulger⁴ that vagus stimulation reduces the refractory period of the auricles. A considerable body of evidence indicates, furthermore, that the duration of electrical systole and of the refractory period vary *pari passu*. It may be concluded, therefore, that acetylcholine reduces the duration of the active state of the mammalian auricle, as well as that of the frog.

Such a quick return to the resting stage may indicate a simple acceleration of the recovery process. If this were the case, subsequent systoles should not differ in other respects from preceding systoles. But this is not the case, for the mechanical response of these beats is greatly reduced, as is the voltage of the accession (*P*) process. In other words, rapid recovery of excitability and conductivity occurs without proper recovery of the ability to contract. Except for a reduction in the length of electrical systole, a similar dissociation of the different functions of muscle occurs in the case of calcium deficiency (Mines⁵).

If the views of Macleod⁶ are correct, an altered state of recovery would be expected to affect the voltages developed during activation. The reason for this inference is that, if the constitution of resting muscle differs from normal, so, in all probability, does the potential difference between it and active muscle. Conversely, if this potential difference is less than normal, as it is after the administration of acetylcholine, it follows that resting muscle (under its influence) is less different from active muscle than is normal resting muscle; it has incompletely recovered. An increase in the duration of the excitation

process (QRS interval) could also cause a reduction in the voltage which is developed, but no significant change in the duration of this process occurs.

No shortening of the electrical response in the ventricle is observed, but there is a definite increase in the height of the regression deflection (T wave). Such a change in the form of the T wave can be accounted for only by alteration in the course taken by the spread of complete recovery, i.e., a local alteration in the duration of electrical systole. It cannot now be demonstrated whether this particular local alteration results from an increase in duration in one locality or a decrease in another. The course of events in the auricle makes the latter seem the more likely, however.

Auricular fibrillation occurs frequently after the administration of acetylcholine. After electrical systole and, of course, the refractory period have been shortened, an extrasystole nearly always gives rise to a series of ectopic beats and frequently initiates an attack of fibrillation. This arrhythmia usually persists until the effect of the drug has worn off to a considerable extent. The occurrence of these paroxysms is probably to be accounted for by a reduction in the duration of the refractory period of the auricles, which facilitates the occurrence of circus rhythm.

Ventricular fibrillation occurred in two of the twenty-eight experiments. The doses of acetylcholine were not larger than those which are usually used. In both cases it persisted long enough to cause death. Since fibrillation of the ventricles is usually attributable to something which alters their refractory period, it is probable that the occurrence of this aberrant rhythm is additional evidence that the drug produces shortening of the refractory period in that part of the ventricles which it affects. The occurrence of this irregularity in cats and dogs under the circumstances of these experiments suggests that a large dose may induce it also in patients.

The most extensive clinical use of acetylcholine and its derivatives has been in the treatment of attacks of paroxysmal tachycardia. Since they have a strong effect on the auricles and only a weak one on the ventricles, it would be surprising if they were not much more efficacious in relieving paroxysmal tachycardia of auricular, than of ventricular, origin. This does, in fact, seem to be true in the few cases of ventricular paroxysmal tachycardia so treated which have been reported. Two patients with, presumably, ventricular paroxysmal tachycardia who were treated by Starr⁷ were not relieved by the drug; nor, in a case reported by Harvey⁸ was acetylcholine efficacious. In the two cases reported by Stern,⁹ mecholine was used, but without benefit.

It is probable that the beneficial effect of the drug in cases of paroxysmal tachycardia depends on its ability to shorten the refractory period, but quinine, which is also useful in the treatment of this condition, has an opposite effect; it lengthens the refractory period. A similar paradox was noticed by Wilson and Wishart¹⁰ in their study of the intravenous use of digitalis, which also shortens the refractory period, for the relief of paroxysmal tachycardia.

They concluded that these drugs apparently terminate attacks in different ways unless they possess a common effect on a still unknown property of cardiac muscle. This alternative probably does not apply in the case of acetylcholine and quinidine, because, as Starr¹¹ has found, quinidine opposes the cardiac action of acetylcholine in animals, and, in patients, a previous dose of quinidine nullifies the effect of acetylcholine on paroxysmal tachycardia. The beneficial effects of the two drugs depend, therefore, on opposite properties

To explain these contradictory effects it becomes necessary to conclude that the desired result is obtained, not by shortening, lengthening, or adjusting the refractory period to some optimal length, but as a mere consequence of changing it. If the refractory period changes, for example, more rapidly than the abnormal mechanism can adjust itself to the new circumstances, the aberrant rhythm will cease. In this connection it is noteworthy that the most effective drugs for this purpose act rapidly and are quickly eliminated. Digitalis is not an exception to this statement, for it seems to be effective only when a large dose is given intravenously. The prophylactic effect of quinidine may depend on the fact that a constant waxing and waning of the refractory period makes the establishment of an abnormal rhythm difficult. On this assumption, a choline derivative with a slower and more prolonged action would be less, rather than more, useful.

The effect of acetylcholine on the mammalian heart is, as this study shows, purely vagomimetic. Although its effect is more intense than that of stimulation of the peripheral end of the severed vagus, it is qualitatively similar. The failure of the drug to have as pronounced an effect on the ventricles as on the auricles is of particular interest.

If the effect of the drug were exercised directly on the myocardium, the ventricles should be as greatly affected as the auricles. If the drug acts not upon the muscle itself, but upon the vagus endings, this selective action is understandable, for it is well known that the ramifications of the vagi reach nearly all parts of the auricles, but only the upper part of the ventricles. This idea is supported by the fact that the frog's ventricle, which is well supplied with vagus endings, is as much affected by the drug as the auricle,¹ and by the observation of Cohn¹² that acetylcholine has no effect upon the chick embryo heart before vagus fibers have grown into it. Since Starr¹¹ has cast grave doubt on Dale's¹³ concept that acetylcholine is effective after the vagus endings are paralyzed, it is probable that acetylcholine is not the effector substance elaborated by the vagus endings, but merely a substance capable of stimulating these endings.

CONCLUSIONS

By recording simultaneous intracardiac pressure pulses and electrograms, it has been shown that the effect of acetylcholine on the auricles is to reduce the force of contraction and the duration of the excited state, and frequently to

produce fibrillation; it has a similar, but much slighter, effect on the ventricles. It is probable that it is more effective in auricular than in ventricular tachycardia and that it is of benefit in this condition because it changes the refractory period rapidly, rather than because it shortens it. It acts on the vagus endings, rather than on the muscle proper.

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A QUANTITATIVE METHOD FOR THE MEASUREMENT OF THE RATE OF WATER LOSS FROM SMALL AREAS, WITH RESULTS FOR FINGER TIP, TOE TIP AND POSTERO-SUPERIOR PORTION OF THE PINNA OF NORMAL RESTING ADULTS

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Most of the methods previously described for the measurement of the rate of sweating from small areas of the human skin have been qualitative (1, 2, 3, 4, 5). A number of quantitative methods have been advocated (5, 6, 7, 8). We found that those that involve the use of an absorbent material, such as filter paper (8) or cloth (6) are inaccurate and non-reproducible. Kuno (5) studied the rate of sweating by using U-tubes filled with calcium chloride to trap the moisture picked up by dry air that had been allowed to flow over a small area of skin. In attempts to use this method it was found that the removal of moisture by calcium chloride U-tubes was not complete at the rate of flow of air necessary to remove the moisture from the skin. Even 1500 grams of finely divided calcium chloride enclosed in a glass tube four feet long did not adequately dry such a stream of air or oxygen. Greuer and Peukert (7) described a method for ascertaining the amount of moisture lost from human skin by measuring variations in the resistance of a semi-conductor. In their method a known area of skin was covered with a shallow chamber roofed by a sodium chloride crystal. The rate of change in resistance of the crystal produced by the water evaporating from the skin and being deposited upon its surface was used as an index of the rate of the elimination of water. Lack of satisfactory standardization of the method made it inadvisable to use it in its present form. A more complete review of the various procedures that have been employed can be found in the publications of Kuno (5), McSwiney (6) and Greuer and Peukert (7). Because of the inadequacy of these methods it became desirable to develop a more accurate procedure. This report concerns itself with a description of, and the results obtained with, the method evolved.

MATERIALS AND METHOD. The apparatus can be divided into three parts: 1, metal cups enclosing the part studied; 2, aluminum coils for the collection of water; 3, a system for providing a stream of dry oxygen.

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1. The chambers for enclosing the finger tips and toe tips were constructed of brass sheeting (0.003 inch thick), cut, shaped and soldered together to form cylinders (diameter about 3 cm.; height, about 4 cm.) (fig. 1 A). The chambers were made of metal instead of cellulose acetate since this substance permits a slow diffusion of water. An opening was made in one end (the proximal end) of the cylinder for the entrance of the part to be studied. Four radial brass tubes (inlets) with an inside diameter of approximately 2 mm. were soldered into the circular wall of the cylinder near the proximal end. A fifth brass tube (outlet) with an inside diameter of approximately 3.5 cm. was soldered to the center of the distal end of the cylinder. A short piece of flexible rubber tubing was fitted over the proximal end of the cylinder. To make an air-tight seal this tubing was

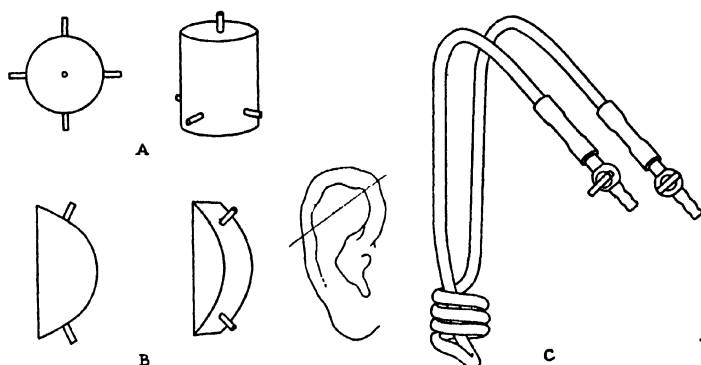


Fig. 1. A—Brass cylinder for enclosing finger tip or toe tip. B—Brass hemi-cylinder for enclosing postero-superior portion of pinna. C—Aluminum coil for collecting water.

chosen to fit closely to, but without constricting, the part inserted for study.

The chamber for enclosing the postero-superior portion of the pinna was also constructed of brass sheeting but was a hemi-cylinder (height about 1.5 cm.; radius about 2.5 cm.) (fig. 1 B). A wide more or less crescent-shaped opening was cut into the flat surface (base) and closed by a rubber membrane. In this an opening was cut, so shaped as to conform accurately to the shape of the pinna lying in the opening. A brass tube (inside diameter approximately 3 mm.) was soldered to each end of the curved wall. One tube served as an afferent and the other as an efferent.

2. A number of aluminum coils (fig. 1 C) for the collection of water was constructed of approximately 1 meter of aluminum tubing (outside diameter 4.8 mm.; inside diameter 3.2 mm.). Each end of the coils was guarded by a metal stopcock. The coils were made to weigh 50 grams to facilitate successive weighings.

3. A stream of oxygen flowing from a tank passed through rubber tubing to an aluminum coil (fig. 2). The coil was placed in a thermos bottle containing a freezing mixture of ethyl alcohol and CO_2 snow. In this coil the oxygen was dried. From the coil it passed in an aluminum tube at least 12 feet long and a four-way distributor into the four inlets of a brass chamber. From the brass chamber, the oxygen, now carrying sweat, proceeded by rubber and aluminum tubing to a second aluminum coil. This coil was also placed in a thermos bottle containing a freezing mixture of ethyl alcohol and CO_2 snow. By means of a system of stopcocks and

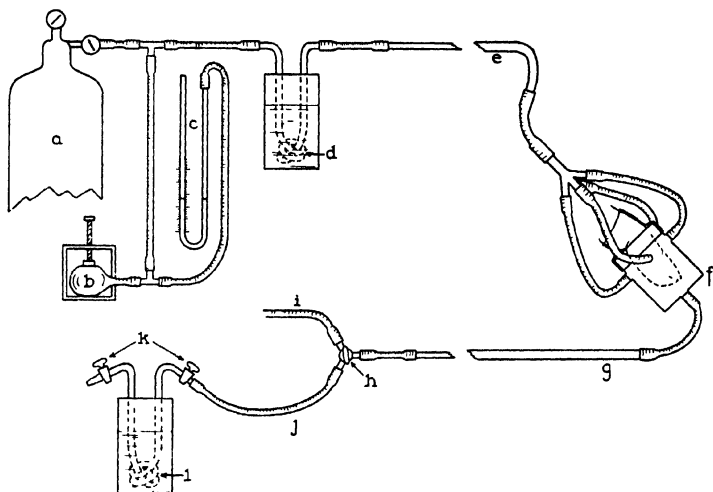


Fig. 2. A complete diagram of the apparatus. *a*, oxygen supply; *b*, pressure bulb controlled by screw clamp, *c*, water manometer; *d*, aluminum coil for drying the oxygen; *e*, aluminum tubing conducting dry oxygen to part; *f*, metal cylinder enclosing part; *g*, aluminum tubing conducting water laden oxygen from part; *h*, 3-way stopcock permitting distribution of oxygen through rubber tubing, *i* or *j*, to alternate water collecting coils; *l*, aluminum water collecting coil with stopcocks, *k*.

rubber tubing it was possible to change the flow to a number of aluminum coils in succession, in order to collect sweat for varying periods of time.

To ascertain the accuracy of the method a brass chamber containing a few drops of water was substituted for the usual cylinder. The air in the chamber was displaced with oxygen and its initial weight obtained. Dry oxygen was passed through the rubber and aluminum tubing for 15 minutes to insure dryness of the system. This oxygen was then permitted to flow through the brass chamber and a weighed aluminum coil immersed in the freezing solution. The water vapor carried from the brass chamber was

deposited in the aluminum coil and the oxygen, having deposited its water, was allowed to escape. After a few minutes the brass chamber was removed. The lengths of tubing which had been connected to the inlet and outlet tubes of the chamber were connected to each other and dried with dry oxygen for 15 minutes. The stopcocks of the aluminum coil were then closed to prevent the escape of water when the coil returned to room

TABLE 1
Data showing the results of the standardization of the method

| SAMPLE NUMBER | WATER INTRO- DUCED INTO THE SYSTEM | WATER COLLECTED BY APPARATUS | DIFFERENCE | ERROR | DIFFERENCE AFTER COR- RECTION OF 0.4 MGM. | ERROR AFTER CORRECTION |
|------------------|------------------------------------------|------------------------------------|------------|----------|----------------------------------------------------|---------------------------|
| | mgm. | mgm. | mgm. | per cent | mgm. | per cent |
| 1 | 69.6 | 71.1 | +1.5 | +2.15 | +1.9 | +2.7 |
| 2 | 49.9 | 49.8 | -0.1 | -0.20 | +0.3 | +0.6 |
| 3 | 47.4 | 46.1 | -1.3 | -2.74 | -0.8 | -1.7 |
| 4 | 31.8 | 31.1 | -0.7 | -2.20 | -0.3 | -0.9 |
| 5 | 31.2 | 30.6 | -0.6 | -1.92 | -0.2 | -0.6 |
| 6 | 28.0 | 27.3 | -0.7 | -2.50 | -0.3 | -1.1 |
| 7 | 21.4 | 21.1 | -0.3 | -1.40 | +0.1 | +0.5 |
| 8 | 20.4 | 20.6 | +0.2 | +0.98 | +0.6 | +2.9 |
| 9 | 20.3 | 19.9 | -0.4 | -1.97 | 0.0 | 0.0 |
| 10 | 17.3 | 17.9 | +0.6 | +3.46 | +0.9 | +5.2 |
| 11 | 15.6 | 15.5 | -0.1 | -0.64 | +0.3 | +1.9 |
| 12 | 14.2 | 13.5 | -0.7 | -4.93 | -0.3 | -2.1 |
| 13 | 14.0 | 13.2 | -0.8 | -5.71 | -0.4 | +2.9 |
| 14 | 12.1 | 11.5 | -0.6 | -4.95 | -0.2 | -1.6 |
| 15 | 12.0 | 12.1 | +0.1 | +0.83 | +0.5 | +4.2 |
| 16 | 12.0 | 11.4 | -0.6 | -5.00 | -0.2 | -1.6 |
| 17 | 11.7 | 10.8 | -0.9 | -7.69 | -0.5 | -4.3 |
| 18 | 7.5 | 6.8 | -0.7 | -9.30 | -0.3 | -4.0 |
| 19 | 7.2 | 6.7 | -0.5 | -6.99 | -0.1 | -1.4 |
| 20 | 5.3 | 5.1 | -0.2 | -3.70 | +0.2 | +3.8 |
| 21 | 4.3 | 3.8 | -0.5 | -1.16 | -0.1 | -2.3 |
| 22 | 3.6 | 2.9 | -0.7 | -19.40 | -0.3 | -8.3 |
| 23 | 3.4 | 2.7 | -0.7 | -20.50 | -0.3 | -8.8 |
| 24 | 3.2 | 2.8 | -0.4 | -12.50 | 0.0 | 0.0 |
| 25 | 2.6 | 2.2 | -0.4 | -15.30 | 0.0 | 0.0 |
| Mean | 18.6 | 18.2 | -0.4 | | | 2.6 |

temperature. The brass chamber and the aluminum coil were both weighed a second time to learn the amount of water which had been lost from the former and the amount gained by the latter. This procedure was repeated for 25 separate measurements (table 1).

The mean loss of water from the brass chambers was 18.6 mgm. and the mean amount of water picked up in the aluminum collecting coils was 18.2

mgm., an error of -0.4 mgm. or -2.2 per cent (table 1). The variation from this mean error was small. Such an error, insignificant for large amounts, became increasingly important as the amount of water deposited decreased. Since the error was about 0.4 mgm. and since we were not in position to learn its source, we corrected our results arbitrarily by the addition of this amount. The correction is applicable in the 15 subjects.

To measure the rate of perspiration, fingers, toes or ears were sealed in their brass cylinders with rubber cement. Since three parts were studied simultaneously, three separate streams of dry oxygen were provided. Leaks were detected by the use of a water manometer. The oxygen flow was adjusted to 300 to 500 cc. per minute in each chamber, pressure not exceeding 3.5 cm. of water. For the first 30 minutes, oxygen being used to dry the systems was allowed to escape. To collect and measure the water eliminated, the oxygen was made to pass through the aluminum coils immersed in the freezing mixture. After a certain time the flow having passed through one group of aluminum coils was directed through a second group. Flow through a succession of coils was carried out for 15 minute intervals for a total of 60 to 90 minutes.

Certain precautions in weighing the coils were taken: 1. To insure uniform dryness before use the inside of the coils was dried by passing room air through them and the outside by blowing room air over them. The coils were then filled with dry oxygen at atmospheric pressure before being weighed. 2. After the collection of the water, the oxygen in the coils having attained room temperature was brought to atmospheric pressure by opening one of the stopcocks momentarily to allow the escape of the excess of oxygen.

The flow of the dry oxygen through the brass chambers did not affect their temperature materially as measured by thermocouples placed within the chambers.

Because of variations in the size of the parts studied, measurements of the surface area of the finger tip, toe tip and postero-superior portion of the pinna were made using methods previously described (9, 10, 11). The formula given by Isbell (9) for measuring the surface area of the finger tip from its volume was found applicable to the toe tip with a maximum error of 3 per cent. The finger tip as used in these studies is defined as that portion of the finger distal to a plane passing through the distal major dorsal and palmar skin creases. The toe tip is that portion of the toe distal to a plane passing through the distal major dorsal and plantar skin creases. The postero-superior portion of the pinna lies above a plane passing at right angles to the lateral surface of the pinna and slightly postero-superior to the portion of the pinna joining the scalp (see fig. 1).

RESULTS. Measurements of the rate of water eliminated from small areas of skin were made of 15 normal white adults (6 males and 9 females)

varying in age from 22 to 52 years. The subjects were studied at various times of the day while resting in bed and covered to satisfy each individual's comfort. The atmosphere of the room was controlled to maintain a temperature of $75^{\circ}\text{F.} \pm 1$ and a relative humidity of 50 per cent ± 3 . The parts for study were adjusted to the level of the heart. The subjects rested for an hour before collections were started. In 7 of the subjects the measurements were repeated after an interval of several days to weeks.

The mean amount of water collected from the right index finger tip of the resting subject was 1.86 mgm. per square centimeter per 15 minutes, the variations ranging from 3.82 to 0.81. The mean value for the right second toe tip was 1.18 mgm. per square centimeter per 15 minutes, the variations ranging from 2.16 to 0.52. The values for the postero-superior portion of the pinna of the right ear were found to possess a mean of 0.48

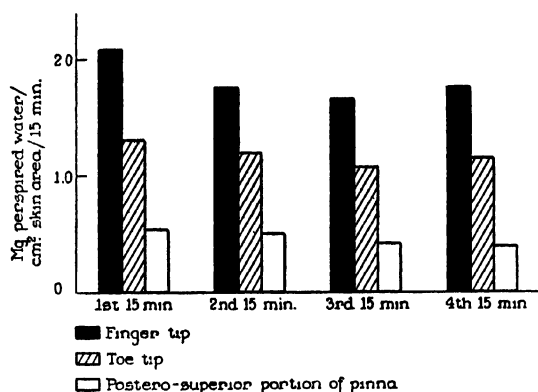


Fig. 3. Proportional rates of water perspired by finger tip, toe tip and pinna during four successive 15-minute periods.

mgm. per square centimeter per 15 minutes, the variations ranging from 0.69 to 0.29. The rate of the loss of water was greater from the finger tip than from the other two parts studied. The ratio of the mean rates of the elimination of water of the finger tips to toe tips was approximately three to two; of finger tips to postero-superior portion of the pinnae, approximately four to one. In two subjects the finger tip to toe tip ratio deviated markedly, being almost one to one. In most of the measurements it was found that the rate of the loss of water in the first 15 minutes was slightly greater than in the succeeding fifteen minute periods when there was a tendency to reach a constant level (fig. 3).

DISCUSSION. The method employed in these observations is open to certain objections; 1, because the atmosphere surrounding the parts studied was different from that surrounding the rest of the body; 2, because the

relative humidity of the room being 50 per cent, while that in the brass chambers was much less, may have tended to increase the rate of evaporation from the surface of the skin. The degree to which the transpiration of water and the activity of sweat glands were influenced by these factors is unknown. The nature of the gas surrounding the parts may have influenced the results but the degree was probably insignificant. In spite of these criticisms the method offers certain advantages. It is simple, accurate, and does not disturb the subject after the parts have been enclosed in the brass chambers. Controlling the flow of oxygen, turning the stop-cocks, changing the collecting coils, weighing and drying the coils, and per-

TABLE 2

Rate of sweating (mgm./sq. cm. surface area/15 min.) in the right index finger tip of 13 normal white resting adults

| SUBJECT NUMBER | AGE | SEX | SURFACE AREA FINGER TIP | FIRST 15 MINUTES | SECOND 15 MINUTES | THIRD 15 MINUTES | FOURTH 15 MINUTES | MEAN FOR 15 MINUTES |
|-------------------|-------|-----|----------------------------------|---------------------|----------------------|---------------------|----------------------|------------------------|
| | years | | sq. cm. | | | | | |
| 1 | 30 | M | 11.64 | 2.56 | 2.27 | 1.72 | 1.63 | 2.05 |
| 2 | 40 | M | 10.64 | 2.27 | 1.84 | 2.07 | 1.97 | 2.04 |
| 3 | 33 | M | 11.47 | 1.83 | 1.31 | 1.31 | 1.74 | 1.55 |
| 4 | 27 | M | 12.06 | 2.67 | 1.92 | 1.44 | 1.41 | 1.86 |
| 5 | 28 | M | 12.94 | 2.26 | 3.82 | 2.13 | 1.74 | 2.49 |
| 6 | 39 | F | 9.81 | 2.75 | 2.08 | 1.78 | 2.01 | 2.16 |
| 7 | 30 | F | 8.77 | 1.38 | 1.37 | 1.40 | 1.74 | 1.47 |
| 8 | 40 | F | 9.04 | 1.64 | 1.97 | 2.01 | 2.03 | 1.91 |
| 9 | 23 | F | 10.25 | 1.95 | 1.83 | 1.88 | 1.87 | 1.88 |
| 10 | 52 | F | 10.17 | 1.01 | 0.93 | 0.81 | 1.12 | 0.97 |
| 11 | 25 | F | 10.51 | 2.81 | 2.03 | 1.51 | | 2.12 |
| 12 | 30 | F | 8.54 | 1.79 | 1.55 | 1.43 | 1.43 | 1.55 |
| 13 | 50 | F | 10.58 | 2.16 | 1.85 | 2.15 | 2.40 | 2.14 |
| Mean..... | | | | 2.08 | 1.75 | 1.66 | 1.76 | 1.86 |
| Max..... | | | | 2.81 | 3.82 | 2.13 | 2.40 | 2.49 |
| Min..... | | | | 1.01 | 0.93 | 0.81 | 1.12 | 0.97 |

forming the other necessary manipulations can be, and were, done far removed from the subject's bed. By modifying the size and shape of the various parts of the system the method can be used to study the rate of elimination of water from many normal or diseased surfaces.

The differences in the rate of loss of water from the parts studied were marked. The rate was two-thirds as rapid in the toe tip as in the finger tip and one-quarter as rapid in the postero-superior portion of the pinna as in the finger tip. The reason for these differences may be physiologic or anatomic or both. Since there are no satisfactory anatomical data (5, 12) on the number of sweat glands in the areas studied it is impossible to decide what the reasons are which account for the difference.

There are marked variations in the rate of water loss in different individuals (tables 2, 3 and 4). The rate of elimination of water from the

TABLE 3

Rate of sweating (mgm./sq cm surface area/15 min.) in the right second toe tip of 14 normal white resting adults

| SUBJECT NUMBER | AGE | SEX | SURFACE AREA TOE TIP | FIRST 15 MINUTES | SECOND 15 MINUTES | THIRD 15 MINUTES | FOURTH 15 MINUTES | MEAN FOR 15 MINUTES | RATE OF SWEATING AS PER CENT OF FINGER TIP RATE |
|----------------|--------------|-----|----------------------|------------------|-------------------|------------------|-------------------|---------------------|-------------------------------------------------|
| | <i>years</i> | | <i>sq cm</i> | | | | | | |
| 1 | 30 | M | 10 99 | 1 09 | 0 79 | 0 69 | 0 70 | 0 82 | 39 |
| 2 | 40 | M | 10 70 | 1 48 | 1 40 | 0 97 | 1 06 | 1 23 | 58 |
| 3 | 33 | M | 10 51 | 1 41 | 1 41 | 1 17 | 1 35 | 1 34 | 90 |
| 4 | 27 | M | 9 97 | 1 15 | 1 18 | 0 82 | 0 91 | 1 02 | 61 |
| 5 | 28 | M | 12 12 | 1 56 | 2 16 | 1 58 | 1 47 | 1 69 | 68 |
| 6 | 39 | F | 10 92 | 1 62 | 1 09 | 0 89 | 1 09 | 1 17 | 52 |
| 7 | 30 | F | 8 81 | 0 81 | 0 81 | 0 52 | 0 96 | 0 78 | 51 |
| 8 | 40 | F | 10 50 | 1 00 | 0 84 | 0 89 | | 0 91 | 43 |
| 9 | 33 | F | 11 23 | 1 02 | 1 21 | 0 90 | 0 94 | 1 02 | 55 |
| 10 | 52 | F | 8 67 | 0 82 | 0 89 | 0 82 | 0 91 | 0 86 | 92 |
| 12 | 30 | F | 8 33 | 1 13 | 0 90 | 1 12 | 1 07 | 1 06 | 70 |
| 13 | 50 | F | 9 17 | 1 35 | 1 30 | 1 29 | 1 49 | 1 33 | 64 |
| 14 | 22 | F | 8 55 | 2 08 | 1 52 | 1 73 | 1 72 | 1 76 | |
| 15 | 30 | M | 10 79 | 1 82 | 1 13 | 1 65 | 1 30 | 1 48 | |
| Mean | | | | 1 31 | 1 19 | 1 07 | 1 15 | 1 18 | |
| Max. | | | | 2 08 | 2 16 | 1 73 | 1 72 | 1 76 | |
| Min | | | | 0 81 | 0 79 | 0 52 | 0 70 | 0 78 | |

TABLE 4

Rate of sweating (mgm./sq cm surface area/15 min.) in the posterior-superior portion of the pinna of 5 normal white resting adults

| SUBJECT NUMBER | AGE | SEX | SURFACE AREA OF PORTION OF PINNA | FIRST 15 MINUTES | SECOND 15 MINUTES | THIRD 15 MINUTES | FOURTH 15 MINUTES | MEAN | RATE AS PER CENT OF FINGER TIP RATE |
|----------------|--------------|-----|----------------------------------|------------------|-------------------|------------------|-------------------|------|-------------------------------------|
| | <i>years</i> | | <i>sq cm</i> | | | | | | |
| 1 | 30 | M | 13 21 | 0 64 | 0 60 | 0 57 | 0 50 | 0 58 | 30 |
| 2 | 40 | M | 13 86 | 0 66 | 0 69 | 0 42 | 0 40 | 0 54 | 26 |
| 3 | 33 | M | 13 02 | 0 50 | 0 44 | 0 41 | 0 42 | 0 44 | 29 |
| 4 | 27 | M | 13 28 | 0 41 | 0 38 | 0 41 | 0 30 | 0 38 | 23 |
| 13 | 50 | F | 13 60 | 0 49 | 0 41 | 0 29 | 0 34 | 0 39 | 16 |
| Mean. | | | | 0 54 | 0 50 | 0 42 | 0 39 | 0 48 | |
| Max.. | | | | 0 66 | 0 69 | 0 57 | 0 50 | 0 58 | |
| Min.. | | | | 0 41 | 0 38 | 0 29 | 0 30 | 0 38 | |

finger tip of subject number 2, for instance, was almost twice as rapid as from subject number 10. Although the rate of the elimination of water

varied within the same individual from time to time the level remained fairly constant. The rates are apparently correlated with the emotional type of the subject. Phlegmatic subjects lost water less rapidly than excitable ones.

SUMMARY

A method is described for measuring the rate of water loss from small surfaces. The method consists in passing dry oxygen through chambers covering the surfaces and then conducting the moisture-containing oxygen through cold aluminum coils. From the difference in weight of the coils before and after the passage of the oxygen, the amount of water lost is learned. The method is accurate to 2.6 per cent. This error can, however, reach 9 per cent when less than 6 mgm. of water are measured, but such low values were not encountered.

The rate of the elimination of water was studied from the right index finger tip, right second toe tip and postero-superior portion of the right pinna of 15 white, normal, resting adult subjects. The mean rate of water loss was found to be 1.86 mgm. per square centimeter per 15 minutes for the finger tips, 1.18 mgm. for the toe tips and 0.48 mgm. for the pinnae. The rate of water loss in the toe tips was approximately two-thirds as rapid as in the finger tips and the rate for the pinnae was only one-quarter as rapid as that for the finger tips.

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THE EFFECT OF TYROSINASE ON EXPERIMENTAL HYPERTENSION

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PLATES 29 AND 30

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Recent advances in the study of experimental arterial hypertension have suggested that the elevated blood pressure results from an increase in peripheral resistance caused by the presence of a pressor substance circulating in the blood stream. This humoral vasoconstrictor is probably released by the kidneys when their blood flow is reduced. Although the nature of the pressor substance is unknown, some have suspected that it might be of relatively simple chemical structure. Victor has found a highly pressor dialyzable material in the anaerobic autolysate of kidneys (1); Page has isolated "angiotonin" from the interaction of renin and a globulin in plasma (2). "Angiotonin," a dialyzable substance, requires, however, "an activator" for its pressor action (3).

Barger found that many phenolic compounds containing an aliphatic amine as a side chain are strong pressor substances, the most potent being aminoethanol catechol, which is closely allied to adrenalin (4). Although other amines may exhibit a pressor action, the most powerful appear to contain a catechol configuration in the molecule.

It appeared possible that the pressor substance responsible for hypertension is a simple pressor amine, released by ischemic kidneys because of an alteration in the action of some system of enzymes requiring oxygen. Justification for this conception is found in the work of Holtz (5), who was able to demonstrate by experiments *in vitro* that while renal tissue was able to decarboxylate tyrosine and di-hydroxyphenylalanine (dopa) in the absence of oxygen, deamination occurred only when oxygen was present. His results suggest, therefore, that an amine oxidase is present in kidneys. When it is remembered that the product of the decarboxylation of di-hydroxyphenylalanine is hydroxytyramine, a pressor substance, and of the deamination di-hydroxyphenylacetic acid, which is inert, this theory becomes tenable. Further substance would be given to this idea if these renal enzymes were not specific for tyrosine and dopa, but acted upon all

TABLE IV
Diastolic Pressure and Heart Weight of Rats
Normal animals

| Rat No. | Weight | Weight of heart | | | Diastolic pressure |
|--------------|--------|-----------------|----------|----------|--------------------|
| | | Expected | Observed | Change | |
| | gm. | gm. | gm. | per cent | mm. Hg |
| P 9 ♀ | 252 | 0.796 | 0.89 | +11 | 90 |
| P 13 ♀ | 286 | 0.875 | 0.97 | +11 | 75 |
| 50 ♀ | 375 | 1.082 | 1.17 | +8 | 90 |
| 43 | 384 | 1.103 | 1.18 | +7 | 88 |
| P 7 ♀ | 326 | 0.975 | 1.03 | +6 | 70 |
| P 16 ♀ | 286 | 0.875 | 0.91 | +4 | 110 |
| P 14 ♀ | 286 | 0.875 | 0.87 | 0 | 86 |
| 34 | 344 | 1.009 | 1.00 | -1 | 104 |
| P 5 | 276 | 0.861 | 0.84 | -2 | — |
| 29 | 350 | 1.030 | 0.98 | -5 | 104 |
| P 8 | 300 | 0.909 | 0.86 | -5 | 92 |
| 28 | 340 | 1.006 | 0.95 | -6 | 100 |
| P 17 | 326 | 0.975 | 0.90 | -8 | 95 |
| 33 | 322 | 0.964 | 0.88 | -9 | 110 |
| P 12 ♀ | 256 | 0.805 | 0.73 | -9 | 42* |
| P 15 ♀ | 286 | 0.875 | 0.79 | -10 | 86 |
| Average. . . | | | | -0.5 | 89.5 |

All rats were males unless otherwise designated.

* Animal in shock when blood pressure was measured.

TABLE V
Diastolic Blood Pressure in Mm. Hg, before and after the Injection of Tyrosinase
Abnormal animals

| Rat No. | Dose | Control | 15 min. after injection | 30 min. after injection | Change at 30 min. | Remarks |
|---------|-------|---------|-------------------------|-------------------------|--------------------|----------------|
| | units | mm Hg | mm Hg | mm. Hg | mm. Hg | |
| H 93 | 225 | 120 | 50 | 70 | -50 | 85 at 24 hrs. |
| H 94 | 450 | 110 | 81 | 65 | -45 | 82 at 70 min. |
| H 95 | 180 | 102 | 50 | 65 | -37 | 70 at 2 wks. |
| H 97 | 450 | 126 | 93 | 85 | -41 | |
| H 100 | 225 | 112 | 52 | 82 | -30 | 60 at 5 days |
| G 104 | 360 | 134 | 86 | 100 | -34 | 92 at 2 wks. |
| G 113 | 225 | 110 | 65 | 69 | -41 | 82 at 98 min. |
| G 115 | 450 | 120 | 102 | 74 | -46 | |
| G 117 | 360 | 132 | 86 | 92 | -40 | 82 at 75 min. |
| G 121 | 225 | 110 | 104 | 80 | -30 | 72 at 80 min. |
| G 122 | 225 | 110 | 100 | 72 | -38 | 62 at 75 min. |
| G 123 | 270 | 128 | 90 | 88 | -40 | 100 at 17 days |
| GN 140 | 450 | 128 | 75 | 68 | -60 | 85 at 75 min. |
| I 9 | 500 | 112 | 62 | 70 | -42 | |
| I 19 | 337 | 112 | 60 | 60 | -52 | |
| I 22 | 180 | 138 | 108 | 96 | -42 | 80 at 70 min. |
| Average | 320 | M = 119 | 79 | 77 | M = -42 σ = 7.6 | |

of heparin was injected intravenously to prevent clotting in the needle of the manometer. It was sometimes found necessary to perform tracheotomy. One subsequent measurement of blood pressure and rarely two could be made by using the left femoral artery.

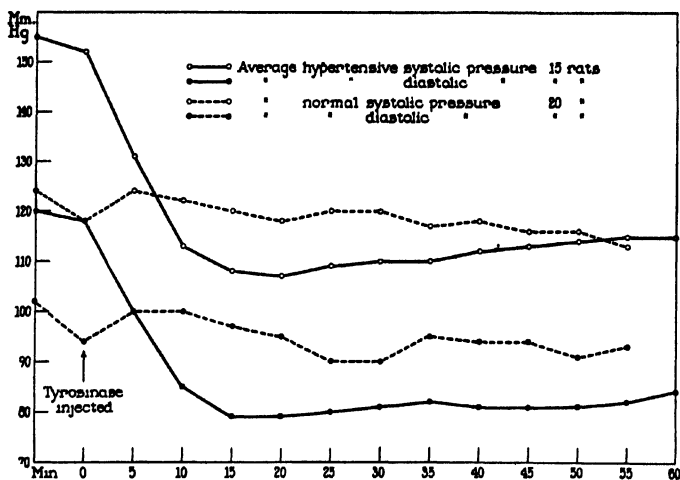
The Effect of Tyrosinase on Hypertensive Rats

The injection of tyrosinase into 16 hypertensive rats was always followed by a fall of blood pressure to normal (Table V). In no case did the blood

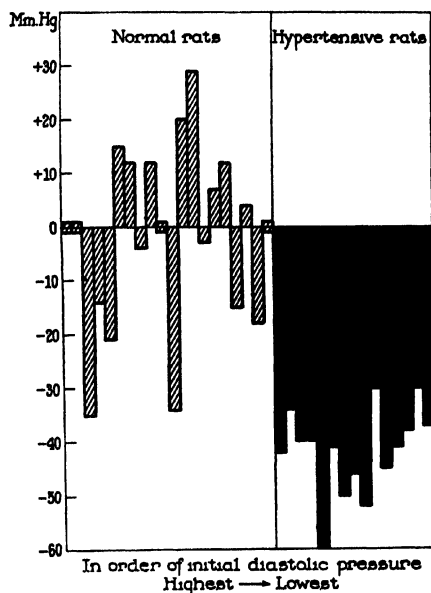
TABLE VI
Diastolic Blood Pressure in Mm. Hg, before and after the Injection of Tyrosinase
Normal animals

| Rat No. | Dose | Control | 15 min. after injection | 30 min. after injection | Change at 30 min. | Remarks |
|-------------|------|-----------------|-------------------------------|-------------------------------|----------------------------|----------------|
| | | units mm. Hg | mm. Hg | mm. Hg | mm. Hg | |
| 22 | 225 | 90 | 121 | 110 | +20 | 110 at 62 min. |
| 23 | 225 | 60 | 70 | 60 | 0 | 60 at 61 min. |
| 24 | 225 | 75 | 70 | 79 | +4 | 79 at 64 min. |
| 26 | 360 | 115 | 95 | 80 | -35 | |
| 27 | 225 | 100 | 100 | 100 | 0 | |
| 28 | 340 | 70 | 50 | 52 | -18 | 55 at 55 min. |
| 29 | 315 | 94 | 70 | 60 | -34 | |
| 31 | 450 | 114 | 100 | 100 | -14 | |
| 32 | 360 | 85 | 118 | 114 | +29 | |
| 33 | 225 | 108 | 110 | 87 | -21 | 112 at 17 days |
| 34 | 225 | 80 | 86 | 92 | +12 | 106 at 9 days |
| 35 | 225 | 105 | 126 | 120 | +15 | 110 at 60 min. |
| 36 | 225 | 100 | 112 | 112 | +12 | 104 at 70 min. |
| 37 | 225 | 136 | 144 | 136 | 0 | |
| 39 | 225 | 90 | 112 | — | — | |
| 40 | 450 | 80 | 65 | 65 | -15 | |
| 41 | 450 | 122 | 126 | 122 | 0 | |
| 42 | 225 | 102 | 104 | 98 | -4 | 98 at 45 min. |
| 43 | 225 | 102 | 110 | 114 | +12 | 110 at 60 min. |
| 45 | 225 | 85 | 86 | 82 | -3 | |
| 56 | 500 | 70 | 70 | — | — | |
| 80 | 450 | 80 | 65 | 87 | +7 | |
| Average.... | 300 | $M = 93$ | $M = 95$ | $M = 93$ | $M = 0$ $\sigma = 16.6$ | |

pressure subsequently rise to previous levels. The intravenous injection of this enzyme into 22 normal rats gave variable results (Table VI). The action of this material was always delayed, no effect being observed, except for a slight initial depression, until 5 to 15 minutes had elapsed (Fig. 1). There was a distinct difference between the responses of normal and of hypertensive animals (Text-figs. 2 and 3). The dose used by the intravenous



TEXT-FIG. 2. The curves represent the averages of the blood pressures of 20 normal and 15 hypertensive rats after the injection of tyrosinase. The values are given in mm. Hg.



TEXT-FIG. 3. The change in the diastolic blood pressure of rats 30 minutes after the injection of tyrosinase. Experiments are shown in the order of the initial diastolic pressure of both the normal and hypertensive animals.

route lay between 180 and 500 units.¹ The only toxic effect observed subsequently was the development of diarrhea in three of the animals, a day or two later. In every hypertensive rat the heart rate was slowed when the injection had taken effect; this reduction was abolished by the administration of atropine without altering the lowered blood pressure. Injections of the enzyme when it had been inactivated by heat (90°C. for 10 minutes) did not affect blood pressure.

Later effects upon blood pressure were observed in five animals, in none of which did hypertension reappear (Table V). These rats did not lose weight and seemed to be healthy in every respect.

The Effect of Tyrosinase on Hypertensive Dogs

Chronic arterial hypertension was induced in dogs by the method of Goldblatt. Usually one renal artery was partially constricted and the other kidney removed, although one dog exhibited persistent hypertension for many months after partial constriction of one renal artery alone. Estimations of blood pressure were made by the use of Hamilton's optical manometer, the needle of which was inserted into the femoral artery. Injections of tyrosinase were made by the intravenous or, in two instances, by the intramuscular route.

Effects somewhat similar to those in rats were seen. Single injections into four hypertensive unanesthetized dogs were followed by marked falls in blood pressure lasting from 3 to 48 hours. Doses varied from 5000 to 20,000 units (10 to 40 mg. protein by dry weight). In three normal dogs there was less change in pressure (Table VII A).

Because the effects of the administration of this enzyme were of relatively short duration, four dogs were treated by daily intravenous injections of 200 units per kilo (Table VII B). The blood pressures of all fell slowly after several days, remaining considerably reduced for several days after the injections were discontinued, and slowly returning to the previous level. Similar treatments given to two normal dogs resulted in one in a rise in the level of blood pressure and in the other in a slight fall (Text-figs. 4 and 5).

Dogs remained healthy and did not lose weight. When large doses were given by the intravenous route, certain symptoms and signs were noted for an hour or two after the injections. These consisted of marked fall in blood pressure, vomiting, diarrhea, and bradycardia. Occasionally there was a rise in body temperature of 1-2°C. 24 hours later bradycardia sometimes persisted although the animals appeared otherwise unchanged. In two experiments bradycardia was abolished after the administration of atropine, although there was no rise in the level of the blood pressure.

¹ One unit has been defined as the amount of enzyme required to cause a solution of hydroquinone and catechol to absorb oxygen at the rate of 10 c.mm. per minute (10).

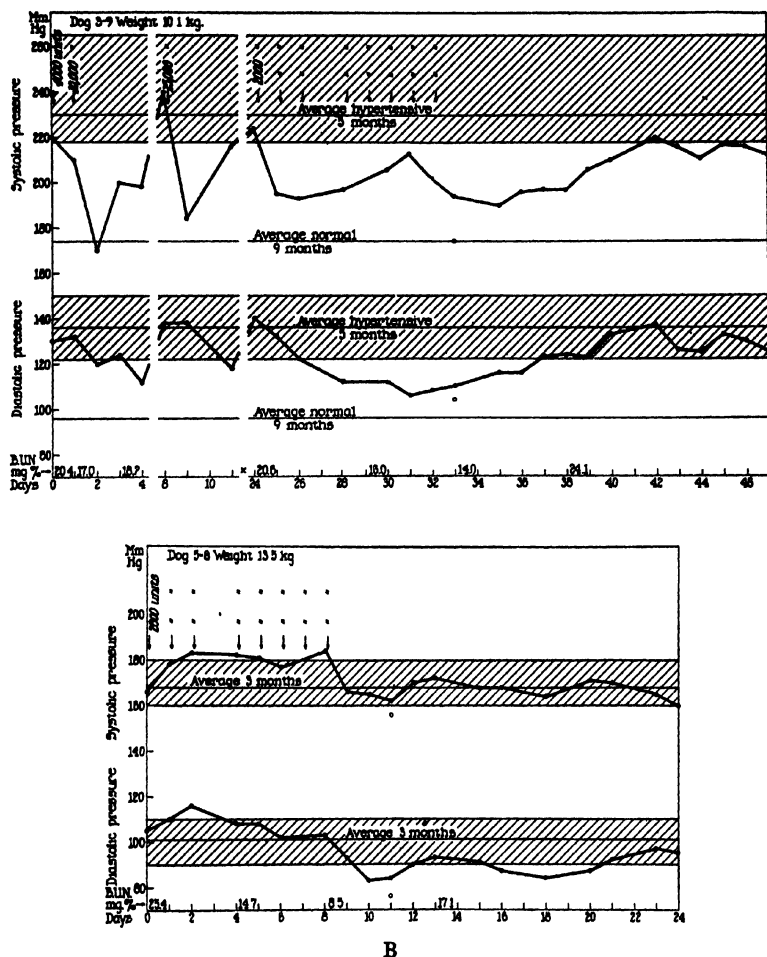
TABLE VII

The Effect of the Injection of Tyrosinase on the Blood Pressure of Dogs

| Dog No. | Date | Dose | Range of blood pressure | | | | Blood pressure after injection | | | | | Change in 24 hrs. | Remarks |
|-------------------|-------------|------------------------|-------------------------|----------------------|--------------------------------------------|--------------|--------------------------------|---------|---------|---------|---------|-----------------------------|---------------------------------------------|
| | | | Before injection | | | At injection | | | | | | | |
| | | | Highest | Lowest | Average | | 3 hrs. | 1 day | 2 days | 3 days | 6 days | | |
| A. Single Doses | | | | | | | | | | | | | |
| -9 | 1940 Mar. 7 | 5,000 | mm. Hg | mm. Hg | mm. Hg | mm. Hg | mm. Hg | mm. Hg | mm. Hg | mm. Hg | mm. Hg | mm. Hg | Goldblatt hy- pertension |
| | | | 228/124 | 190/95 | 202/111 | 220/122 | 160/106 | 160/104 | 182/106 | | 190/126 | -60/-18 | |
| | Apr. 16 | 6,000 | | | | 235/124 | 190/110 | 178/102 | | | | -57/-22 | |
| | 17 | 15,000 | | | | 178/102 | | 194/96 | 194/104 | 224/111 | 222/102 | +16/-6 | |
| | 23 | 15,000* | | | | 224/104 | 170/92 | 177/94 | | 200/90 | 225/114 | -47/-10 | |
| | July 31 | 13,500 | | | | 198/106 | 176/99 | 198/102 | 165/74 | | | 0/-4 | |
| -9 | Apr. 16 | 6,000 | 265/159 | 218/122 | 230/136 | 218/124 | 200/133 | 210/132 | | | | -8/+8 | Goldblatt hy- pertension (unilateral) |
| | 17 | 15,000 | | | | 210/132 | | 168/120 | 200/125 | 198/114 | 240/138 | -42/-12 | |
| | 23 | 15,000 | | | | 240/138 | 168/120 | 180/130 | | 218/120 | 225/120 | -60/-8 | |
| 5-2 | Apr. 16 | 6,000 | 240/145 | 190/104 | 209/118 | 218/124 | 144/110 | 178/122 | | | | -40/-2 | Goldblatt hy- pertension |
| | 17 | 15,000 | | | | 178/122 | | 165/116 | 175/108 | 195/130 | 214/122 | -13/-6 | |
| | 23 | 15,000* | | | | 214/122 | 170/100 | 175/112 | | 208/114 | 190/124 | -39/-10 | |
| 5-6 | Apr. 16 | 6,000 | 178/101 | 136/76 | 155/90 | 152/76 | 165/110 | 130/78 | 178/96 | 172/96 | 178/98 | -22/+2 | Normal |
| -1 | Oct. 10 | 16,500 | 225/122 | 191/90 | 196/108 | 225/122 | 157/122 | 210/108 | | 219/97 | 185/92 | -15/-14 | Normal with elevated blood pressure |
| 6-8 | Oct. 10 | 16,500 | 202/120 | 180/98 | 192/110 | 201/120 | 210/130 | 219/110 | | 193/110 | 185/102 | +18/-10 | Normal with elevated blood pressure |
| B. Multiple Doses | | | | | | | | | | | | | |
| Dog No. | Date | Initial blood pressure | Daily dose | Number of injections | Blood pressure, days after first injection | | | | | | | | Remarks |
| | | | | | 7 days | 8 days | 12 days | 14 days | 18 days | 21 days | 25 days | | |
| 2-9 | 1940 May 9 | mm. Hg | units | | mm. Hg | mm. Hg | mm. Hg | mm. Hg | mm. Hg | mm. Hg | mm. Hg | Goldblatt hyper- tension | |
| | | 192/124 | 2,600 | 8 | 204/110 | 194/108 | 180/104 | 166/88 | 192/104 | 200/122 | 228/120 | | |
| 3-9 | May 9 | 204/140 | 2,300 | 8 | 216/126 | 218/124 | 174/104 | 192/110 | 196/114 | 216/124 | 220/110 | Goldblatt hy- pertension | |
| 5-2 | May 9 | 190/130 | 4,200 | 8 | 188/114 | 176/110 | 156/96 | 164/102 | 188/102 | 220/128 | 208/110 | Goldblatt hy- pertension | |
| 5-3 | May 9 | 226/144 | 3,000 | 8 | 184/110 | 204/136 | 184/108 | 200/110 | 190/120 | 216/124 | 230/136 | Goldblatt hy- pertension | |
| 5-8 | May 9 | 166/96 | 2,800 | 8 | 194/112 | 164/106 | 156/76 | 190/98 | 160/88 | 175/82 | 160/95 | Normal | |
| 6-1 | May 9 | 186/126 | 2,500 | 8 | 240/148 | 210/126 | 194/110 | 182/110 | 200/112 | 204/116 | 190/92 | Normal | |

* Intramuscularly.

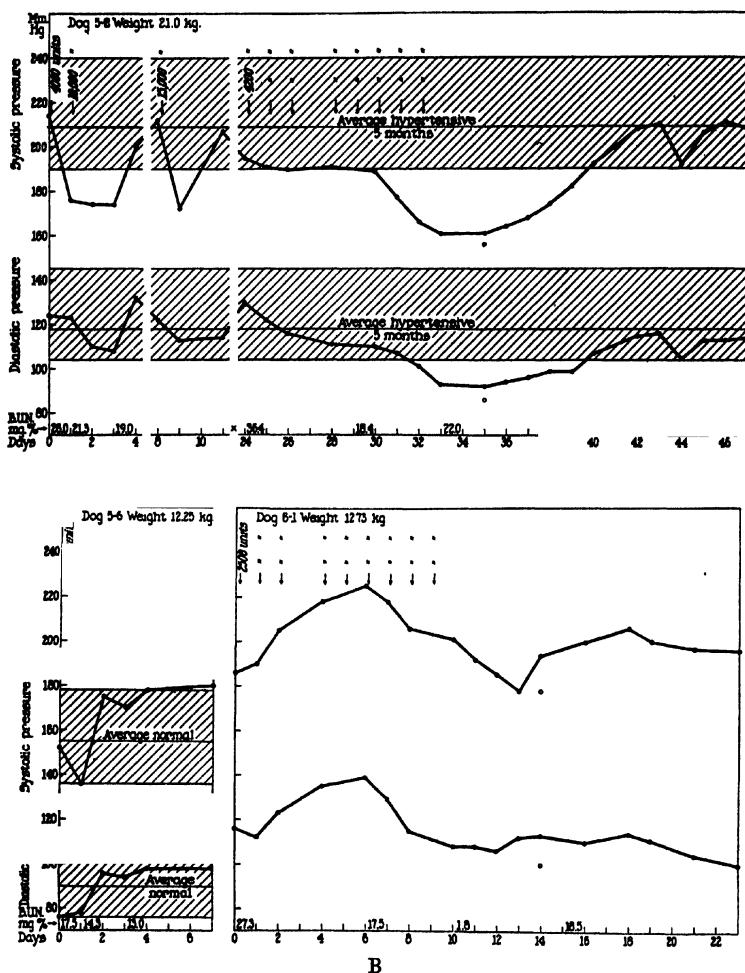
Renal function was measured roughly by the amount of urea nitrogen in the blood. In every instance values were less after the injection of tyrosinase (for example, see Text-figs. 4 and 5). The clearance of urea was



B

TEXT-FIG. 4. The effect of the injection of tyrosinase on the blood pressure of two dogs. A, dog 3-9, hypertensive. B, dog 5-8, normal. The upper curve indicates systolic pressure, the lower, diastolic. The hatched area represents the difference between the highest and the lowest single readings during the control period. Averages are shown. All doses are in catechol units. B. U. N. means urea nitrogen in the blood. From the point marked X the curves are smoothed by the method of running averages of three. The lowest levels reached are shown by circles.

estimated in each of six dogs when daily injections were being given and showed no deviation from the normal. Albuminuria and hematuria when present did not change while dogs were being treated.



TEXT-FIG. 5. Notations same as Fig. 4. A, dog 5-2, hypertensive. B, dogs 5-6 and 6-1, normal.

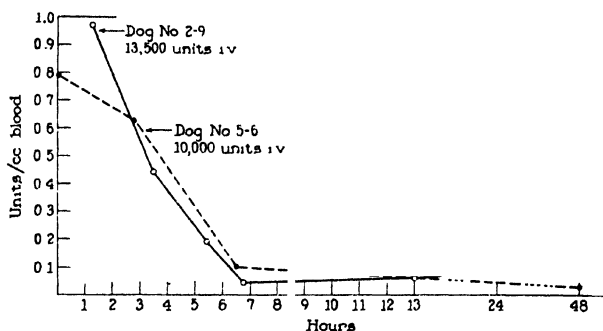
The Duration of the Presence of Tyrosinase in the Blood Stream

After single intravenous injections, tyrosinase was detected in the blood of dogs for 24 hours (Text-fig. 6). No trace of this enzyme has been found

in the blood of dogs or of human beings after subcutaneous or intramuscular injections. As yet, no correlation between the amount of tyrosinase present in the blood and the duration of the fall in blood pressure has been established. Although the enzyme is active in dogs when given by the intramuscular route its presence cannot be demonstrated in the blood.

The Mode of Action of Tyrosinase in Hypertension

Attempts were made to ascertain the mode of action of this enzyme in animals, especially as regards its effect upon various pressor substances.



TEXT-FIG. 6. The concentration of tyrosinase in the blood of two hypertensive dogs at various intervals of time after intravenous injection. The amount was measured in the Warburg apparatus. Values obtained after 7 hours are not accurately determinable, but indicate that traces of enzyme are still present. The value obtained at 48 hours showed a doubtful trace.

25 rats and eight dogs, both hypertensive and normal, were used in brief experiments.

Renal blood flow was measured in dogs by the method of Schroeder and Steele (11), using a thermostromuhr. Blood pressures were recorded by Hamilton's manometer.

Six solutions were used: *Renin* was prepared by Dr. Alfred E. Mirsky and concentrated to a point at which 0.1 cc. consistently produced a pressor response in rats weighing 300 gm. or more. The dose of this material in normal dogs amounted to 1.0 cc. The solution of *tyrosinase* contained 500 units per cc. The solution of *catechol-hydroquinone* (hereafter called C-H) contained 5.0 mg. hydroquinone and 0.1 mg. catechol per cc., buffered in a phosphate solution at pH 6.5, a standard substrate for the enzyme. *Adrenalin* consisted of the usual 1:1000 solution diluted with normal saline according to the amount required. The solution of *tyramine* hydrochloride contained 4.0 mg. per cc. *Angiotonin* was obtained from Dr. Irvine H. Page; 0.05 cc. was one "rat dose" raising the diastolic pressure 50 mm. Hg after intravenous injection.

Tyrosinase and Renin.—When a single dose of renin which had not been freshly dialyzed was shaken with a single dose of tyrosinase for 20 minutes,

it was found that the renin was completely inactivated. When freshly dialyzed renin was used, the response of the blood pressure of dogs and rats was lessened but not abolished. The addition to this mixture of the solution of catechol-hydroquinone resulted in complete inactivation of the renin, C-H itself having no effect when mixed with renin alone (Table VIII). The addition of whole blood to a renin-C-H or to a renin-tyrosinase mixture resulted in marked fall in blood pressure.

Experiments were performed in the Warburg apparatus to ascertain whether tyrosinase acted directly upon renin, or acted through the medium of some other substance. Renin which had stood for several weeks in the cold was shaken with tyrosinase; absorption of oxygen occurred, suggesting that a substrate for tyrosinase was present. No oxygen was absorbed when renin which had been freshly dialyzed was treated in the same manner. A mixture of fresh renin, tyrosinase, and C-H absorbed oxygen (owing to the presence of C-H, a substrate) and was inactive on the blood pressure. In the first instance, therefore, some product of the deterioration of renin acted as a substrate for the enzyme and allowed inactivation of renin. Inactivation likewise occurred when C-H had reacted with tyrosinase.

Tyrosinase and Angiotonin.—When tyrosinase and angiotonin, amounting to one rat dose of each, were shaken at room temperature for 20 minutes and injected intravenously in rats, the mixture was found to have about one-third of the pressor activity of angiotonin alone. The addition of catechol did not change the result. But when 0.1 cc. of rat plasma or rabbit serum was added to tyrosinase and angiotonin, the resultant mixture, after shaking, was found to be completely inactive on the blood pressure (Fig. 2 A). Substitution of gelatin for serum resulted in partial inactivation. These results were observed in five experiments. As was the case with adrenalin, the injection of tyrosinase before or immediately after the administration of angiotonin resulted in a modified pressor response.

When tyrosinase (two and one-half units) and angiotonin (one rat dose) were shaken together in the Warburg apparatus, oxygen was absorbed slowly, and the resulting mixture was found to have lost none of its pressor activity. The addition of a small amount of serum resulted in more rapid and complete absorption of oxygen, and this mixture was completely inactive on the blood pressure of rats (Fig. 2 B). Tyrosinase and serum, or tyrosinase and gelatin, did not cause absorption of oxygen, and were inactive on the blood pressure. Several hours of shaking in the Warburg apparatus were necessary to cause complete inactivation of angiotonin by small amounts of tyrosinase and serum.

Tyrosinase and Other Pressor Substances.—Tyrosinase inactivates adre-

TABLE VIII

The Action of Various Mixtures of Tyrosinase and Renin on the Blood Pressure of Normal Rats

| Solution | Number of experiments | How treated | Color change | Pressor effect* |
|---------------------------------------|-----------------------|---------------------------------------------------------|--------------|------------------------------------|
| | | <i>room temperature</i> | | |
| Renin, old | 10 | Shaken, 15 min. | 0 | ++++ |
| Renin, fresh | 5 | Shaken, 15 min. | 0 | ++++ |
| { Renin, old Tyrosinase | 5 | Shaken, 15 min. | 0 | 0 |
| { Renin, fresh Tyrosinase | 3 | Shaken, 20 min. | 0 | ++ |
| { Renin, old Tyrosinase C-H† | 2 | Shaken, 15 min. | Dark | 0 |
| { Renin, fresh Tyrosinase C-H | 3 | Shaken, 15 min. | Dark | 0 |
| { Renin C-H | 2 | Shaken, 15 min. | 0 | ++++ |
| Tyrosinase | 2 | Shaken, 15 min. | 0 | 0 |
| C-H | 3 | Shaken, 15 min. | 0 | 0 |
| Whole rat blood | 3 | Shaken, 15 min. Standing, 1 hr. | 0 0 | 0 ± |
| { Renin C-H Blood | 3 | Standing, 10 min. Shaken, 10 min. Shaken, 20 min. | 0 0 0 | ++ ----- (died) ----- (died) |
| { Renin C-H Blood Tyrosinase | 2 | Shaken, 15 min. | 0 | ----- (died) |
| { Renin Blood Tyrosinase | 3 | Shaken, 20 min. | Dark | ----- (died) |
| { Tyrosinase Blood | 2 | Shaken, 20 min. | Dark | ----- (died) |

The amount of each solution given was 0.2 cc.

* + indicates rise of blood pressure; - indicates fall.

† C-H = catechol-hydroquinone mixture.

naline *in vitro* almost instantaneously. Tyramine is likewise inactivated although less rapidly. When tyrosinase had been given to a dog or rat, the subsequent injection of tyramine or adrenalin resulted in a modified response, characterized by less elevation of the blood pressure of shorter duration, and sometimes by transient depression of blood pressure after the pressor effect was over.

A quantity of the pressor material obtained from the anaerobic autolysate of renal tissue, prepared by Dr. Joseph Victor, was used in various experiments. Tyrosinase was found to render this material inactive both for dogs and rats. When it was shaken with tyrosinase at room temperature, a depressor response upon injection was noticed in all of six rats and two normal dogs. The presence of catechol-hydroquinone solution was not necessary for this change in effect. The injection of tyrosinase into an animal was found to modify the pressor response of this material when injected subsequently; similarly, the injection of tyrosinase at the height of the pressor response resulted in a fall in blood pressure. In one hypertensive dog, the injection of tyrosinase was found to abolish both the hypertensive state and the further elevation of blood pressure which had resulted from the administration of Victor's material.

Renal blood flow in four normal dogs, measured by the thermostromuhr, was at first reduced after the injection of tyrosinase, and then increased. The reaction of the blood flow to adrenalin by the ischemic kidney was reversed in two experiments, vasoconstriction being replaced by vasodilatation. Hypertension which had resulted from partial constriction of the renal artery and the injection of adrenalin (11) was abolished.

Reactions.—Various reactions followed the intravenous administration of tyrosinase. Anesthetized rats, whether normal or hypertensive, consistently exhibited bradycardia 5 to 15 minutes after the injection, the lowered cardiac rate remaining while the rats were observed (up to 2 hours) and sometimes persisting for 24 hours. A few animals exhibited diarrhea for a day or two after injection. Anesthetized dogs with hypertension developed bradycardia a few minutes after injection. Unanesthetized dogs, normal and hypertensive, exhibited vomiting, diarrhea, tenesmus, and slowing of the pulse, 5 to 30 minutes after injection, lasting one to two hours. The intestines of one animal were seen to be markedly contracted. It was also noticed that excited dogs quieted while the injection was being given. Reactions of this nature were greater in hypertensive dogs, sometimes altering their general behavior; normal ones were less affected. These events did not occur if atropine had been injected. Tyrosinase, in rats and dogs, acted therefore like a cholinergic drug. Little or no change was seen in the behavior of normal rabbits, guinea pigs, or monkeys after intravenous injection, but the blood pressure was not estimated.

The Antigenicity of Tyrosinase

The preparation of tyrosinase used is a good antigen in rabbits. A strong antiserum was prepared and precipitins were detectable at an antigen dilution of 1:400,000. When a solution of tyrosinase was precipitated by this serum, most (9/10ths) of the activity of the enzyme was removed. The precipitate was, however, readily soluble in water and upon solution the enzyme became completely active.

Six guinea pigs were sensitized to tyrosinase by repeated intraperitoneal injections. Subsequent intravenous injections failed to produce signs of anaphylaxis. Sensitized rabbits likewise showed no signs of anaphylaxis after intravenous injection.

The Effect of Other Enzymes on Hypertension

Two enzymes having the property of attacking the guanidine linkage in such compounds as arginine, guanidine, and creatinine were injected into 14 rats and four dogs. *Arginase* obtained from Dr. René J. Dubos and prepared by him from soil bacteria was found to raise blood pressure in hypertensive animals while that of normal ones was less affected. *Arginase* prepared from livers was found to have a similar action, although less pronounced. Both preparations were far from pure, and no conclusions can be drawn from these results.

DISCUSSION

From these experiments there is little doubt that the injection of this preparation of tyrosinase in suitable doses into rats and dogs exhibiting experimental hypertension of the renal variety results in reduction of the blood pressure. Furthermore, there is a difference in the response of normal animals, their blood pressure being less or not at all affected. Although the possibility exists that the hypotensive action of this enzyme is a non-specific one, the most probable explanation of this phenomenon is that some substance present in the hypertensive state is altered. Because tyrosinase acts only on compounds containing a mono- or ortho-di-hydroxybenzene structure in the molecule, the substance which is changed must contain a phenolic group of this nature.

Two possibilities come to mind: the pressor substance itself is inactivated by or through the medium of the enzyme, or some other compound, probably phenolic, is altered to form a depressor substance. From what is known of pressor substances in arterial hypertension the first explanation is more likely; there is no evidence for the second.

There are at least three possible ways in which tyrosinase may act. First, all compounds containing catechol or phenolic configurations are probably oxidized by the enzyme to quinones. This change from the benzenoid to the quinoid configuration is accompanied by an alteration in the physiological action of the compound concerned, as is well illustrated in the case of adrenalin and tyramine. In this way tyrosinase may destroy some as yet unknown phenolic pressor substance which is responsible for hypertension.

A second possibility is that the orthoquinones produced by the action of tyrosinase on phenolic substances have special properties. These orthoquinones, being powerful oxidizing agents, may themselves destroy some pressor substance of lower oxidation-reduction potential in a manner analogous to the destruction of ascorbic acid by orthoquinones (12). These substances themselves can oxidize amines.

The third explanation is that adrenalin is destroyed. Arterial hypertension can be produced in brief experiments by very small amounts of this hormone if one kidney is ischemic. Adrenalin in these amounts is a powerful renal vasoconstrictor in dogs (11). Destruction of adrenalin may allow renal vasodilatation. In that event the blood pressure would fall.

By whatever method tyrosinase acts, it appears to have an action specific for arterial hypertension of the variety produced in these experiments. Furthermore, it inactivates various pressor substances. The part that these materials play in the existence of hypertension is not known, but each behaves as a pressor substance conceivably responsible for bringing on elevation of the blood pressure.

It was shown that *renin* was inactivated by tyrosinase under certain conditions, that is, when catechol or when some product of the deterioration of renin was present. Tyrosinase acts upon catechol to form orthoquinone, which is an oxidizing agent. It is likely, therefore, that the inactivation of renin depends upon the non-specific action of another substance. The significance of an event such as this in the bodies of animals is unknown. If renin were concerned in hypertension it might be inactivated in this manner.

Under certain conditions *angiotonin* is also inactivated by tyrosinase, that is, when serum is present. This reaction does not depend upon the presence of catechol. Although the preparation of angiotonin used was acted upon directly by the enzyme, the reaction was complete only after the addition of serum. Angiotonin according to Page (3) must be activated by some substance present in serum. These results suggest that the product of the activation of angiotonin is a phenolic substance.

Victor's substance, likewise, in the preparations obtained, was inactivated directly by tyrosinase, suggesting that this material also contains a phenolic configuration. The inactivation of adrenalin and tyramine is accomplished by direct action of the enzyme.

Light is thrown on at least one part of the action of tyrosinase by the nature of the various reactions which follow intravenous injection. These effects suggest that tyrosinase acts as a cholinergic drug. This enzyme, however, alters sympathicomimetic substances. The more likely explanation, therefore, is that the sympathetic nervous system is depressed.

There is no explanation for the lowering of the urea nitrogen in the blood of dogs. This change was seen consistently, and did not depend upon a change in amino acids. The least that can be said is that renal insufficiency did not accompany the lowered blood pressure.

The antigenic action of tyrosinase presents a difficult problem. Although it does not occasion anaphylactic shock in guinea pigs, and dogs developed no signs of sensitivity even after 5 months of intermittent use, antibodies (precipitins) have been demonstrated in human beings after subcutaneous injection. Further studies on this subject, both in animals and in human beings, are in progress.

From these studies it becomes obvious that the use of tyrosinase, an enzyme specific in altering phenolic compounds, effectively combats arterial hypertension induced by various methods in rats and in dogs. The use of this enzyme in human beings is now being investigated.

CONCLUSIONS

1. The intravenous injection of tyrosinase, a phenolic oxidase obtained from mushrooms, consistently lowers the blood pressure of rats made hypertensive by three different methods, while on the average not affecting the blood pressure of normal animals.

2. The intravenous and intramuscular injection of tyrosinase lowers blood pressure in dogs made hypertensive by the method of Goldblatt, while affecting that of normal ones to a less extent.

3. Tyrosinase inactivates renin, angiotonin, Victor's pressor substance, adrenalin, and tyramine *in vitro*, and alters the response of the blood pressure to these substances in rats and dogs.

4. Renal function of hypertensive dogs is not depressed when the blood pressure is lowered by tyrosinase.

5. Tyrosinase is an effective substance for combating experimental arterial hypertension. It is probable that some phenolic substance present in hypertension is altered.

After this paper was submitted for publication, a second sample of angiotonin was received from Dr. Page, and the effect of tyrosinase upon it ascertained. Serum was not necessary in this instance for complete inactivation of its pressor action by small amounts of tyrosinase. Amounts of the enzyme as small as 0.2 units abolished the pressor effect of one "rat dose" of this solution of angiotonin, and caused approximately equal absorption of oxygen in the presence and absence of serum.

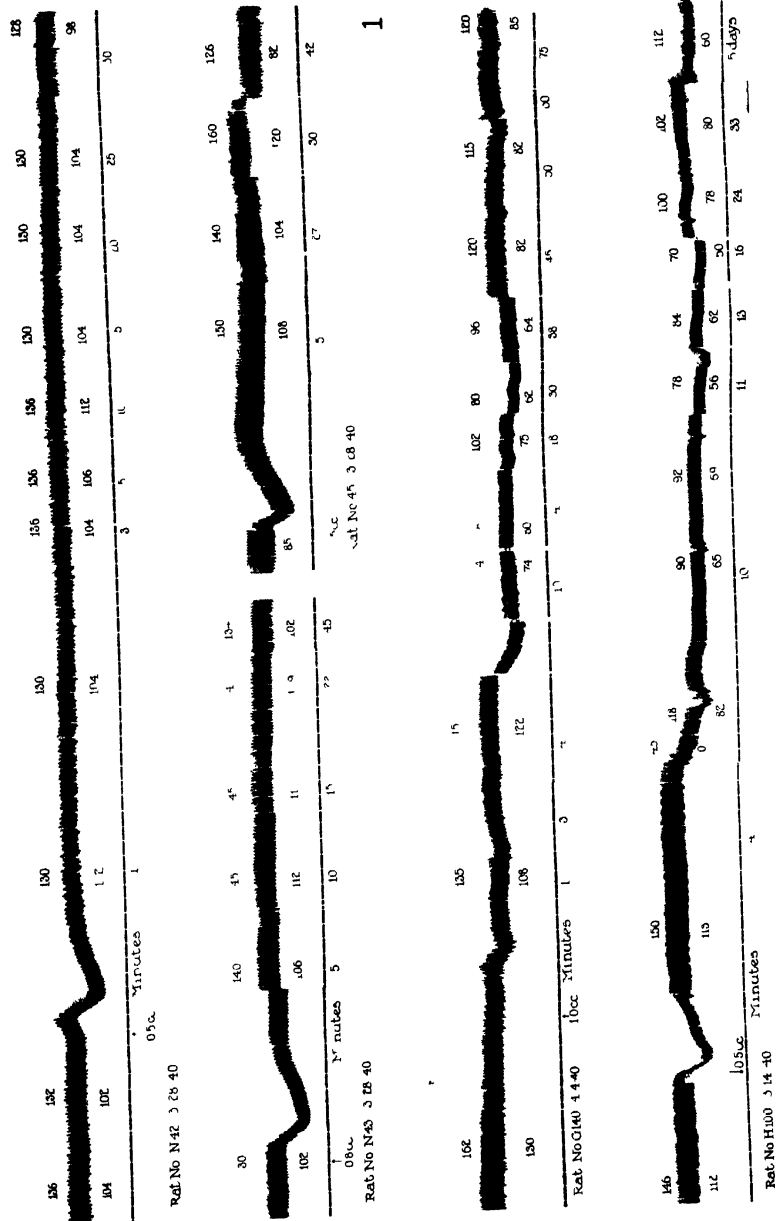
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EXPLANATION OF PLATES

PLATE 29

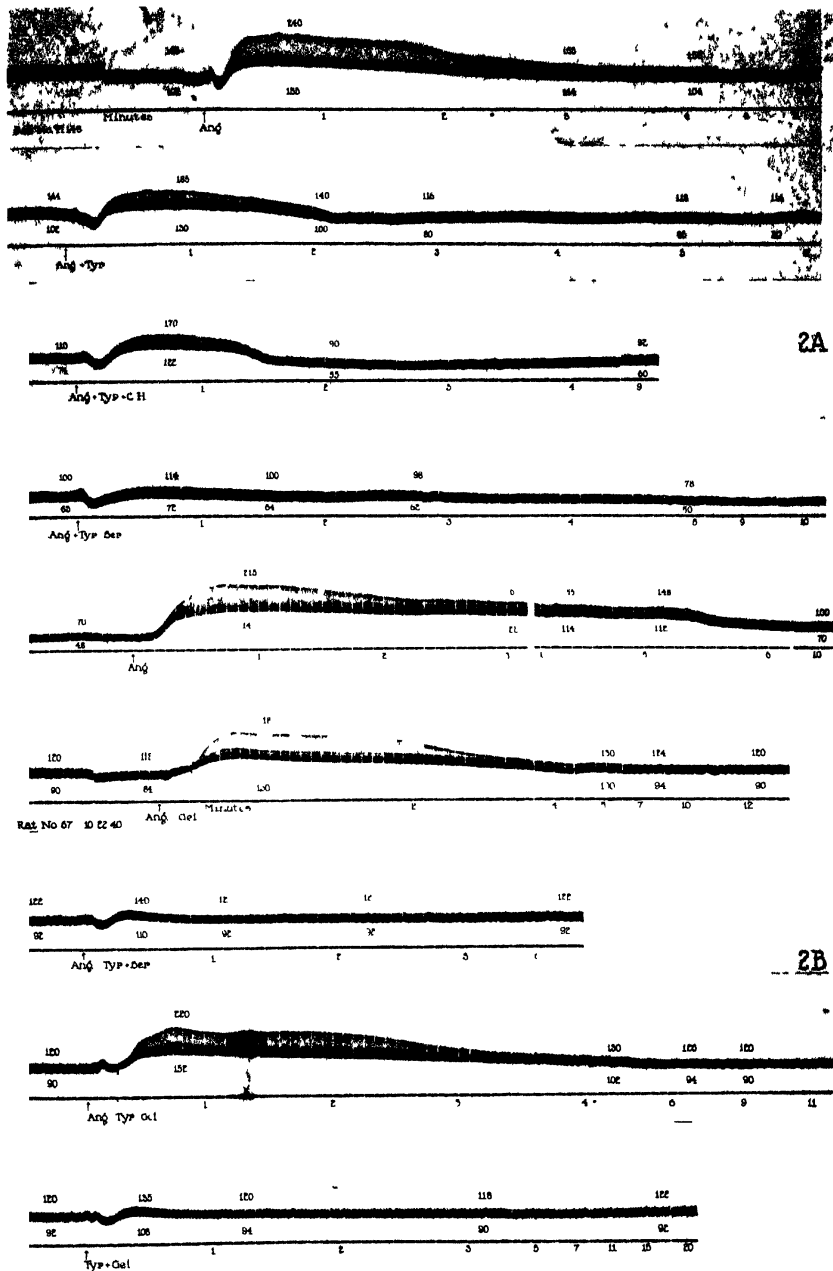
FIG. 1. The effect of the intravenous injection of tyrosinase in rats. The curves are records of blood pressure as photographed with Hamilton's manometer; the upper figures show the systolic, the lower the diastolic pressure. Arrows indicate points of injection. The time is in minutes. Rats 42, 43, and 45 are normal; rat G 140 was made hypertensive by the Goldblatt technique, and rat H 100 by the production of hydronephrosis.



(Schroeder and Adams. Effect of tyrosinase on hypertension)

PLATE 30

FIG. 2. The inactivation of angiotonin by tyrosinase. A, rat H 148, hypertensive. The first curve shows the effect on blood pressure of one rat dose of angiotonin. The pulse pressure has increased. One rat dose of angiotonin and 500 units of tyrosinase, shaken at room temperature for 20 minutes were then administered. There was a decrease in the activity of the former. The third curve shows the effect of the mixture when catechol was added. Angiotonin was still active. In the fourth curve, a mixture of angiotonin, tyrosinase, and serum was injected. Very little activity on blood pressure resulted. The last curve again shows the effect of angiotonin alone. The rat was made hypertensive by the production of unilateral hydronephrosis; notice the fall in the level of blood pressure after the first injection of tyrosinase. B, rat 67, normal. The inactivation of angiotonin by small amounts of tyrosinase. The first curve shows the effect on blood pressure of one rat dose of angiotonin and 6 mg. gelatin which had been shaken in the Warburg apparatus. The second demonstrates the effect of the same amount of angiotonin, 0.1 cc. serum, and 2.5 units of tyrosinase similarly treated. Angiotonin was inactive. In the third curve is seen the effect when gelatin was substituted for serum. The last curve shows the effect of tyrosinase and gelatin, an inert mixture.



IN VITRO CONDITIONS FAVORING ECDYSIS AT THE END OF THE FIRST PARASITIC STAGE OF HAEMONCHUS CONTORTUS (NEMATODA)

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The pathogenic nematode, *Haemonchus contortus*, which lives as a parasite in the fourth or true stomach (abomasum) of its host, is found in ruminants throughout the world. In common with related forms, such as hookworms, this "twisted wireworm" has four larval stages, the termination of each of which is marked, morphologically, by ecdysis, much as in insect growth.

The first two of the larval stages are obligate free-living forms, feeding on bacteria in the host feces in which the eggs have been conveyed outside. At the end of this early two-stage development the larvae are, for the first time, infective, and are unable to feed or advance further until they reach the host in which they are to be parasitic. In the case of *H. contortus* the infective larvae migrate from the sheep feces to blades of grass and other forage plants, and are thus conveniently placed for ingestion by the grazing animal. Until passively transferred back to their parasitic environment, they are to a certain extent protected by the presence of a cuticular sheath, which forms a loose coat around the infective 3rd stage larva within. It is possible that this sheath may be lost early after ingestion, perhaps in the ruminant's mouth. All later development in this species is believed to take place on the wall of the abomasum. The parasitic phases include development of the 3rd stage larva to its ecdysis freeing the 4th stage, growth (3 to 6 fold increase in length) and development of the 4th stage to its ecdysis freeing the young adult, with final growth (another 3 to 6 fold increase in length) and development of the adult to the reproductively mature worm.

Attempts have been made by Glaser and Stoll (1938a) to bring this entire life cycle to pass in test tubes under conditions of bacterial sterility. "Culturing" of the normally free-living stages of strongyloid worms of this sort naturally in feces is not difficult. As a step toward culture of the parasitic stages Glaser and Stoll (1938b) first reared these free-living stages without bacterial contamination in agar-yeast-liver extract media. Efficient infective larvae were developed, and confirmed as such by their ability to parasitize sheep. As far as further bacteria-free culture was concerned, such sterilely

grown infective larvae appeared to offer no advantage, however, over growing them to the infective stage in the usual nonsterile fashion and then chemically sterilizing them (Glaser and Stoll, 1940). Utilizing this preliminary procedure, it was possible to initiate parasitic growth and carry it forward in culture to the end of the 2nd parasitic (4th larval) stage (Glaser and Stoll, 1938c). In such cultures no adults were found, and advanced forms were usually encountered dead or moribund. Of considerable significance nevertheless was the fact that the block at the end of the free life had been broken under in vitro conditions and true parasitic development induced.

With the problem offering a certain refractory character at this level, the author undertook to explore more particularly some of the factors influencing initiation of the parasitic phase itself (i. e. the changes culminating in ecdysis at the end of the 3rd stage) as a step in a better evaluation of the rôle of different culture conditions and ingredients. The present report deals primarily with salt solutions and liver extract, indicating those of choice, with the selection of their optimum concentrations and conditions of test.

Materials and Methods

Source of Infective Larvae

To secure and maintain a supply of *H. contortus* infective larvae, a pure infection with these parasites is established (Stoll, 1929) in a lamb or sheep reared helminth-free after the method of Smith and Ring (1927). Such infections can be maintained serially, each lasting for a variable number of weeks, depending on condition of the host, number of larvae introduced into the sheep, etc. There is no increase in the number of parasites within the host. Infections of suitable size are established by administering by mouth a single dose of 60 to 200 larvae per kg. of host weight, i. e., 3000 to 5000 infective *Haemonchus* larvae to an animal of 25-50 kg.

Various methods have been devised to get good yields of infective larvae from sheep feces, but the simplest one, which is approximately the natural one, turns out to be best. This is to collect feces from the infected sheep directly into paraffined paper boxes, add a small amount of water, cover and set aside on the laboratory table (Sarles, 1932). Free-living nema contaminants are avoided by preventing contact of fecal pellets with the rump wool of the host, and the use of distilled water. At room temperature 7-10 days are required for larvae to develop to the infective stage. With 50-90 grams of fecal pellets thus "cultured" in a half-pint box, yields of infective larvae, isolated after 10-20 days, range from 30-85 per cent of the number of eggs. Larvae may thus be secured in abundance, if the source animal has an infection yielding 2000 to 4000 eggs per gram (Stoll, 1930).

The larvae are freed from the fecal mass by using the Baermann isolation technic. Due to the abundance of mold spores in the fecal "culture" at the

time of isolation, Baermann funnels are best placed in a room distant from the one where the later sterile technics are undertaken. If the larvae secured at isolation are found to contain undeveloped or dead specimens, attempts to sterilize them result unsatisfactorily.

Preparation of Larvae for in Vitro Experiments

With infective larvae in good condition, the method of Glaser and Stoll (1940), using Labarraque's solution of sodium hypochlorite, has been routinely successful in freeing them from the sheaths which mark the termination of the 2nd stage and securing the 3rd stage forms bacteriologically sterile. A single simplification has been developed in this technique. As one step, the larvae are repeatedly allowed to settle through columns of sterile tap water in order to wash from them the microorganisms not killed by the hypochlorite, and those additional bacteria gradually released from their intestinal tracts. Instead of using for this, long tubes with constricted ends which are later flame-sealed after filling with water, sterile bacteriological test tubes (18 X 160 mm.) are now employed. These permit the use of 10 ml. of sterile tap water through which the larvae settle to the bottom of the tubes. Following gravity washing as the larvae fall faster than organisms freed from them, the supernate is withdrawn and the larvae are transferred to successive tubes for further washing in the same fashion. About three times as many sterile water washings are required in using the test tubes. The entire process is carried out at room temperature.

Sometimes as much as half the original number of larvae are lost through clumping and lagging in sedimentation during the sterilizing procedure, but 20,000 to 40,000 or more may be brought through the 3-day process successfully in one lot. After sterilizing, and until needed, they are held in the refrigerator at 5° C in a shallow layer of dilute Tyrode solution in 50 ml. Erlenmeyer flasks. All larvae are tested for sterility before use in experiments.

So far no consistent differences have been found in the ability of the larvae to undergo their first parasitic ecdysis in vitro as regards their age in culture before chemically sterilizing them, or their age in the refrigerator afterward,—provided vigorous larvae are used. Larvae for inoculation of culture tubes have not been employed unless 95 to 100 per cent of a fair sample of the sterile lot are alive and wriggling. In one test of the relation of the "physiological age" of sterile larvae to their ability to undergo ecdysis later, no difference could be noted between one half of a lot which had been refrigerated 5 days, and the other half of the same lot which had been incubated in a flask in shallow, dilute Tyrode solution at 38½° C for 5 days, the lots then tested in parallel in Tyrode-liver extract and Ringer-liver extract media.

The use of exsheathed, infective larvae free from contamination underlies all subsequent experimentation, which in every detail (except final examination of larvae after incubation) is carried out under conditions of bacteriological

sterility. For those whose previous experience with parasites has taken account of only helminthological sterility, the exclusion of contaminating bacteria and fungi in cultures will have numerous disappointments. Due to the large number of tubes to be filled with media and inoculated in single experiments, the avoidance of windy days in beginning an experiment has, for instance, proved a saving precaution in these tests.

When tubes have been sealed after inoculation, "Parafilm" has been used over the cotton plugs trimmed down even with the top of the tube.

Preliminary Observations

As mentioned, the infective *Haemonchus* larva at the end of the free-living period and in its 3rd stage is enclosed in a loosely fitting sheath which originally formed the cuticula of the 2nd stage larva. In the sterilizing process with sodium hypochlorite solution the larva is first set free from this sheath, and then the sheath itself dissolves. Under conditions of natural parasitism this larva would have been ingested by the sheep while still within the 2nd stage sheath. The freed 3rd stage larva thus constitutes the bridging form which comes at the end of life in the open and at the beginning of life in the host. But unless external conditions are appropriate, no further change takes place in such a larva, and it either dies after exhaustion of its stored nutriment or succumbs earlier to some hazard of its environment. Lapage (1935), despite his ability to sterilize and exsheath the infective larvae of *Haemonchus* and other strongyloid forms, reported only 10 parasitic ecdyses in more than 1200 chemically exsheathed 3rd stage larvae studied, and "the occurrence of these third ecdyses did not appear to be related to the composition of the media in which they occurred."

Under proper conditions, however, the larvae do proceed to develop to the 4th stage. Then one observes that:

1. As the 4th stage develops it is seen to become gradually free from the 3rd stage sheath, first at head and tail, eventually throughout its length. This is evidently not so much due to enlargement of the sheath as to a gradual decrease in size of the young parasite. In one series of measurements, in which unmodified 3rd stage larvae averaged 570μ in length, ten 4th stage larvae, still ensheathed, measured 415μ , their intact sheaths 580μ , representing a decrease in length of over one-fourth. The impression is gained that in tubes showing ecdysis most promptly, the pre-ecdysis decrease in size is less.

Lapage (1935) observed that "parasitic larvae are often retracted in their sheaths," but refers to it as "shrinkage and distortion," "an osmotic effect." The decrease seems better interpreted as due primarily to the utilization of stored food material during the period of initial parasitic development, which, as a sort of "pupation" ending in ecdysis, results in a smaller but more advanced larva. As shown later, ecdysis occurs in three-salt Ringer solution containing

only inorganic constituents, and to a very small degree even in distilled water. Exogenous food sources are thus evidently not necessary to initiate the parasitism, as Lapage (1935) speculated and the present studies appear to prove. While Veglia (1916), examining recently parasitized sheep, noted "little or no growth in the third stage" of *Haemonchus*, the present observation of initial decrease is made more readily *in vitro* than *in vivo*. Miller (1939) found a decrease in size averaging about 1/5th in length (from 1.6 to 1.3 mm.) in the trematode *Postharmostomum laruei* "after it has been in the [mouse] host 30 hours." Ferguson (personal communication) has observed a similar average decrease in length of about 1/5th in another trematode, *Posthodiplostomum minimum*, during its first few days in the bird host. It is possible this is a common phenomenon in the first stage of helminthic gastrointestinal parasitism.

2. The mouth of the larva undergoes marked modification, the smoothly rounded anterior end becoming noticeably blunt, even when observed under low power magnification. This is due to the development of the provisional buccal capsule. To quote Veglia again (1916): "The object of this period of life seems to be the formation of a mouth apparatus adapted to piercing the mucosa of the stomach." The change is clearly shown in Fig. 1, where comparison may be easily made between the rounded anterior end of the sheath representing 3rd stage morphology, and the blunt mouth of the 4th stage larva within. (The curved line connecting the two is the cuticular lining of the earlier esophagus.)

There is no change in the posterior end of the emerging 4th stage larva except for the tail to be held slightly at an angle to the main axis of the body. This is in contrast to the change from the long attenuated kinked tail characterizing the 2nd stage to the stubbier tip of the 3rd stage, as illustrated in an earlier article (Glaser and Stoll, 1940).

3. When the morphological changes are completed ecdysis occurs through the escape of the young 4th stage breaking through the old sheath. Break-through characteristically occurs not at the old mouth opening, as Lapage (1935) also points out, but back from the anterior end about 1/8th of its length (Fig. 2). Occasionally the anterior end of the old sheath is invaginated, due to the effort of the larva to pull away from the cuticular lining of the old esophagus, shown in Fig. 1, but usually the cast sheath is a clean shadow of the 3rd stage form. If by chance a cotton fibril or other piece of sterile debris happens to be present in the culture tube, the sheaths are caught in numbers about it, as in Fig. 3.

According to Veglia, 70 per cent of the larvae may be free 4th stages by the 48th hour after infection of the sheep. *In vitro*, although ecdysis is occasionally observed within 48 hours, the period is longer for most of the larvae. Up to now, 70 per cent by the 4th day would classify as an exceptional *in vitro*

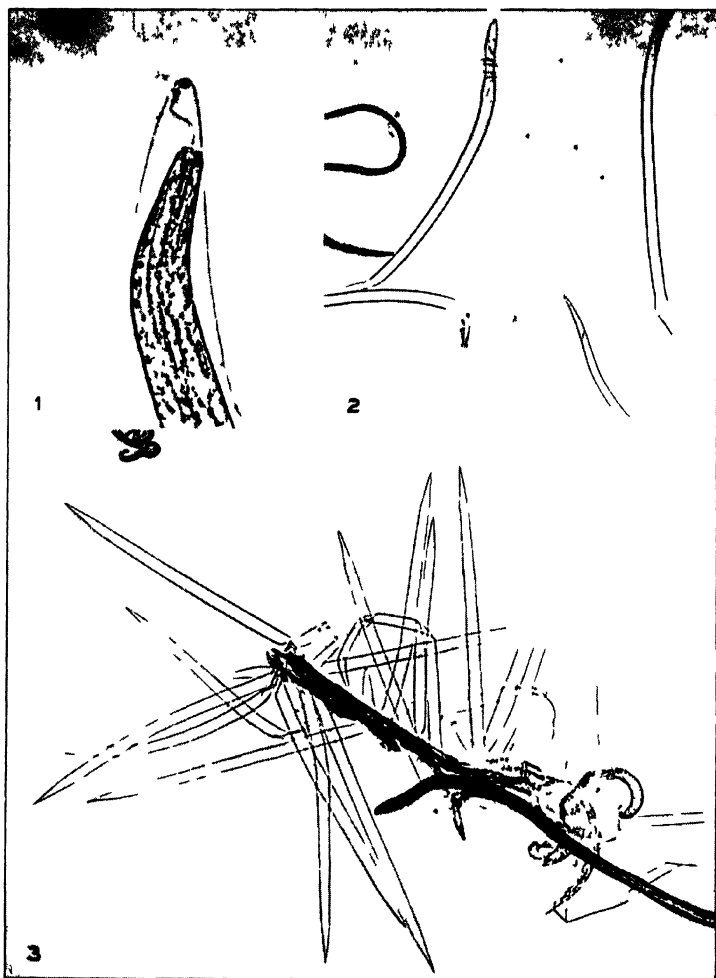


FIG. 1 Enlarged view of anterior end of *Haemonchus* larva showing provisional buccal capsule of 4th stage, within first parasitic sheath. Cuticular lining of 3rd stage esophagus is still attached to the old mouth. Occasionally on cecidysis this region of sheath is invaginated due to efforts of the larva to free itself from the esophageal cuticula.

FIG. 2 Cast sheaths, first parasitic cecidysis of *Haemonchus* the usual point where larva breaks through visible a slight distance from anterior end. Not infrequently this anterior portion breaks off completely as a temporary 'cap' for the 4th stage larva.

FIG. 3 First parasitic sheaths of *Haemonchus* tangled on piece of sterile debris in culture tube.

(Photomicrographs by J. A. Carlick)

result, but ecdysis has been observed to that degree in one tube examined on the 4th day.

4. If the young 4th stage larvae are not in media which contain appropriate nutriment they soon die. Generally speaking the exsheathed 3rd stage larvae introduced into culture tubes are very hardy. If parasitic development does not occur they may persist at incubator temperatures for several weeks, though in gradually decreasing numbers (see Graph 1a), growing more and more pale as the food stored in their intestinal cells is used up. This is a familiar observation concerning infective larvae of strongyloid forms, especially hookworm. The newly emerged 4th stage gives evidence of being physiologically distinct from the 3rd stage in its inability to survive without exogenous nutriment under the same conditions. As the studies here reported do not deal with nutrient media, the paradoxical criterion gradually arose that—barring losses due to contamination or specific toxic factors such as sodium chloride or strong liver extract—good conditions for ecdysis and high death rates ran more or less in parallel.

This ability of the 3rd stage larvae to make the transition to the 4th stage apparently entirely on their endogenous food reserves has proved a fortunate circumstance, inasmuch as it has permitted distinguishing factors which induce development into parasitism, from the factors favorable to further growth afterward. The degree to which ecdysis takes place thus becomes a measure of the favorableness of the environment for parasitism, and of the conditioning factors being tested.

Experimental Results

Salt Solutions

Two experiments are illustrated. Exper. 1, in two parts, indicates the relative desirability of Tyrode, Ringer, and physiological salt (normal saline) solutions in various dilutions, as tested in unsealed tubes; Exper. 2 deals with Tyrode solution alone in sealed tubes.

The Tyrode solution used (Parker, 1938) contains 8.0 gm. sodium chloride, 0.2 gm. potassium chloride, 0.2 gm. calcium chloride, 0.1 gm. magnesium chloride, 1.0 gm. sodium bicarbonate, 0.05 gm. sodium acid phosphate, and 1.0 gm. glucose in one liter of distilled water. In making up Tyrode, if each ingredient is dissolved in the water before adding the next, no cloudiness results. Sterilization is by candling through a Berkefeld N. The three-salt Ringer solution (Parker, 1938) can, on the other hand, be sterilized by autoclaving and contains 9.0 gm. sodium chloride, 0.42 gm. potassium chloride, and 0.25 gm. calcium chloride in one liter of distilled water. The normal saline used was autoclaved 0.85 per cent sodium chloride.

In the first part of Exper. 1 comparisons of the salt solutions and their dilutions are made on the basis of the amount of parasitic ecdysis occurring

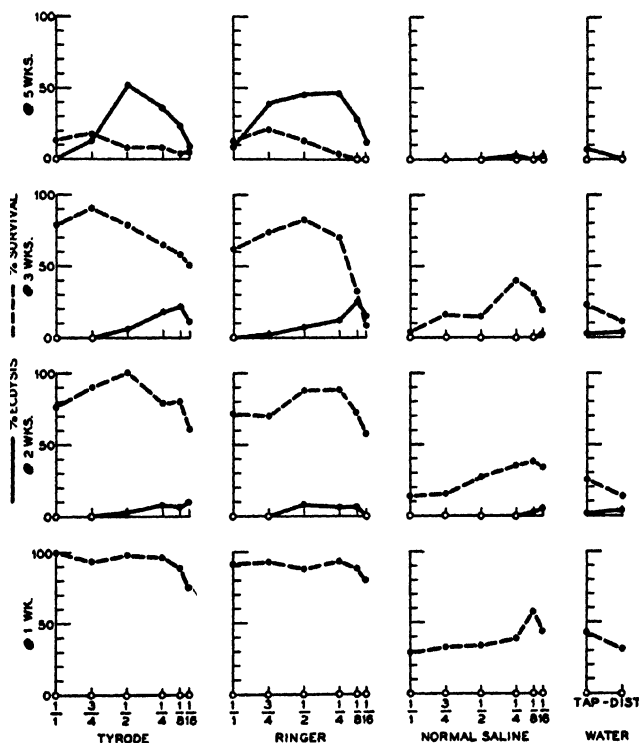
with *Haemonchus* larvae in the incubator at 38½°C, in the second part, by the degree of survival of the larvae in the refrigerator at 5°C, a temperature at which ecdysis has not been observed.

Exper. 1A. To small test tubes, 100 × 10 mm, were added 2 ml. amounts of Tyrode, Ringer, and sodium chloride solutions at the usual 1/1 physiological concentration, and dilutions of 3/4, 1/2, 1/4, 1/8, and 1/16. Tubes with similar amounts of tap water and distilled water were used as controls. These dilutions produced a series in which the salt concentrations were respectively 160, 120, 80, 40, 20, and 10 millimols per liter. (Normal saline at 0.85 per cent represents 145 mM/L. Its dilutions were made to reproduce the above millimolecular series, except full concentration which represented 145 rather than 160.) The tubes were inoculated with an average of 55 sterile, exsheathed 3rd stage *Haemonchus* larvae, and closed with a cotton plug but not otherwise sealed. There was some evaporation from the tubes during incubation, and those continuing in the experiment after the 2nd week, and also after the 3rd week, were opened with sterile precautions and about 1/2 ml. distilled water added to restore original depths of approximately 35 mm.

At the end of 1, 2, 3, and 5 weeks the larvae in each tube were examined and counted, as were the empty sheaths. The larvae, being poor swimmers, are always in the bottoms of the tubes with the sheaths, and all can be withdrawn in a single drop. The percentages of larvae which had undergone parasitic ecdysis (i.e., number of sheaths in relation to whole number of larvae in tube) are shown in Graph 1A, as well as the percentage of surviving larvae at each examination. The results are plotted for the 80 individual tubes, not as averages.

No ecdysis was found in any tube the first week, but there was a perceptible beginning by the end of the second week, absent however in the 1/1 and 3/4 concentrations of Tyrode and Ringer, and in all but the most dilute saline. Ecdysis in saline, tap water, and distilled water, did not later exceed the minute amount apparent at two weeks. By the end of the 3rd week, while 1/1 Tyrode and Ringer still showed no sheaths, there was an increase to over 20 per cent in the 1/8 dilutions. This had further increased but little by the end of the 5th week in the most concentrated and dilute, but in the mid-range of dilutions from a third to a half of the larvae had emerged as 4th stage forms. The relatively good ecdysis rates occurring sooner in the 1/8 dilutions of Tyrode and Ringer solutions are suggestive of results reported by Hogue (1919) who found that diluted Locke-Lewis solutions acted as a stimulant for tissue cultures. The fact that ecdysis rates at Tyrode and Ringer dilutions more nearly isotonic for *Haemonchus* larvae are eventually greater in degree than at the hypotonic 1/8 dilution is further suggestive of Miss Hogue's observation that in hypotonic Locke-Lewis solution tissues grew more rapidly but did not live so long as in isotonic media.

These young 4th stage larvae soon died, a fact reflected in the survival curves in Tyrode and Ringer solution, which represent 3rd stage larvae which have not yet undergone ecdysis. The poor survival of the larvae in saline is obviously unrelated to ecdysis, representing instead the marked toxicity of sodium chloride without the benefit of antagonistic ions, as is usual with bio-



GRAPH 1A. Comparison of Tyrode, Ringer, and sodium chloride solutions in unsealed tubes in production of first parasitic ecdysis of exsheathed infective *Haemonchus* larvae. The millimolar concentrations of the Tyrode and Ringer dilutions range from 160 at 1/1, to 10 at 1/16; "normal saline" dilutions are arranged to parallel these, except 1/1 which is 145. Controls in tap and distilled water.

logical material. The marked decrease in number of survivors at one week, with all dead in 5 weeks, is also true of the controls in tap water and distilled water.

Exper. 1B. As a parallel to Exper. 1A, a duplicate set of tubes was similarly inoculated from the same batch of larvae and placed in the refrigerator at 5°C. Evaporation from these tubes was slight, scarcely reaching at the end of 3

months that in the incubator after 2 weeks. Because of the known ability of *H. contortus* to remain alive under refrigerated conditions, additional tubes were included so that examinations could be made after the 5 week period.

Due to the sluggishness of larvae examined while cold, the tubes were removed from the refrigerator 1 to 3 hours before examination. (See below the special treatment given a second set at the 3 month interval.) Neither ecdysis nor development preliminary to ecdysis was observed in any tube. The data on survival are plotted for the 140 individual tubes in Graph 1B.

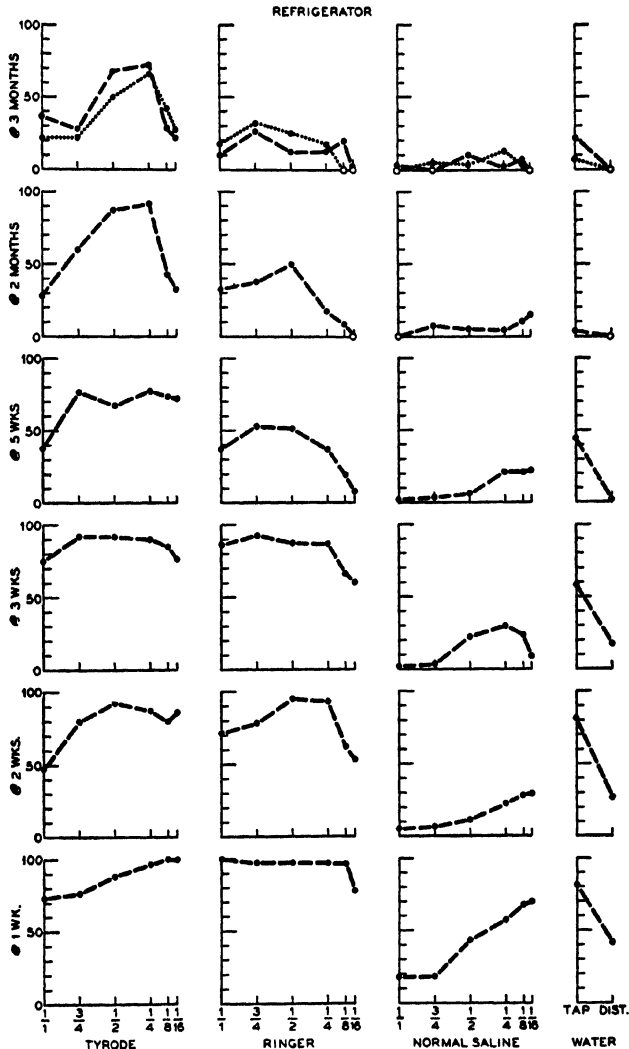
Even by the end of the first week the death rate in normal saline was greatly increased, over either Tyrode or Ringer, although a small percentage lived for 3 months. Survival in general was better in the more dilute sodium chloride than in the "normal" concentration, although distilled water alone is an improvement for a period. Larval *Haemonchus* evidently better resist the hypotonic action of pure water in the first few weeks, than the toxic sodium ions in most of the saline dilution. Judged by the survival data the laboratory tap water* is so much better that it classifies as the substitute, which it frequently is, of a dilute "balanced" salt solution, such as Ringer.

Between Tyrode and Ringer solutions there is little distinction in the first 3 weeks' results, but there is at 5 weeks and thereafter greater survival in the Tyrode. Experience gained in other tests has indicated that this advantage of Tyrode as compared to Ringer solution, which shows more distinctly in Graph 1B than in Graph 1A, is on the whole characteristic with *H. contortus*.

While survival was anticipated at 3 months, it was a surprise to find it so good in Tyrode. To test whether larvae stored this long at low temperatures would respond to in vitro conditions to undergo ecdysis, a set of the tubes still remaining was examined in the following manner. A new series of tubes was prepared, 2 ml. each, with a Tyrode-liver extract medium known to be effective in producing ecdysis (the 0.10 gm. per ml. concentration illustrated in Exper. 3 was used). A set of the refrigerator tubes was then opened under sterile conditions, and larvae from each were transferred to Tyrode-liver extract medium. The new tubes were then incubated for 4 days and examined. An insufficient number of the live larvae remained from the original Ringer, saline, and water sets to give a test, but from the 6 original Tyrode tubes ecdysis had taken place in from 3 to 37 per cent (average 21 per cent) of the larvae still alive on the 4th day of incubation (which represented 2 to 16 per cent, average 7 per cent, of all the larvae, dead and alive, in the tubes). Refer-

* This water was from 200 foot wells. A routine commercial analysis made during the course of these studies showed, in grains per gallon: "total hardness 3.1 (temporary 1.9, permanent 1.2); total (bicarbonate) alkalinity 4.7 (both hardness and alkalinity in terms of CaCO_3); chlorides (Cl) 0.8; sulphate (SO_4), less than 1.5; iron, none; pH 6.7." Tap water, autoclaved, is used during the exsheathing and sterilizing of the infective larvae. Baermann isolations are done with tap water at body temperature.

ence to Exper. 3 will show that a similar Tyrode-liver extract concentration in that experiment produced 30 per cent ecdysis, with 70 per cent of the larvae surviving, on the 4th day. This demonstration of ability to undergo ecdysis



GRAPH 1B. Survival of exsheathed infective *Haemonchus* larvae in graded dilutions of sterile Tyrode, Ringer, and normal salt solutions in refrigerator, 5°C, with controls in tap and distilled water.

after refrigeration thus attested fairly good physiological condition of the larvae, and biologically confirmed the survival curve at 3 months in Tyrode solution.

The results of the two parts of Exper. 1 are in essential agreement. Judged either by ecdysis at the end of the first parasitic (3rd larval) stage in the incubator, or simple survival of exsheathed infective larvae at a temperature (refrigerator) too low to permit ecdysis,—

1. Balanced salt solutions, such as Tyrode and Ringer, present more favorable conditions than sodium chloride solution alone, tap water, or distilled water.

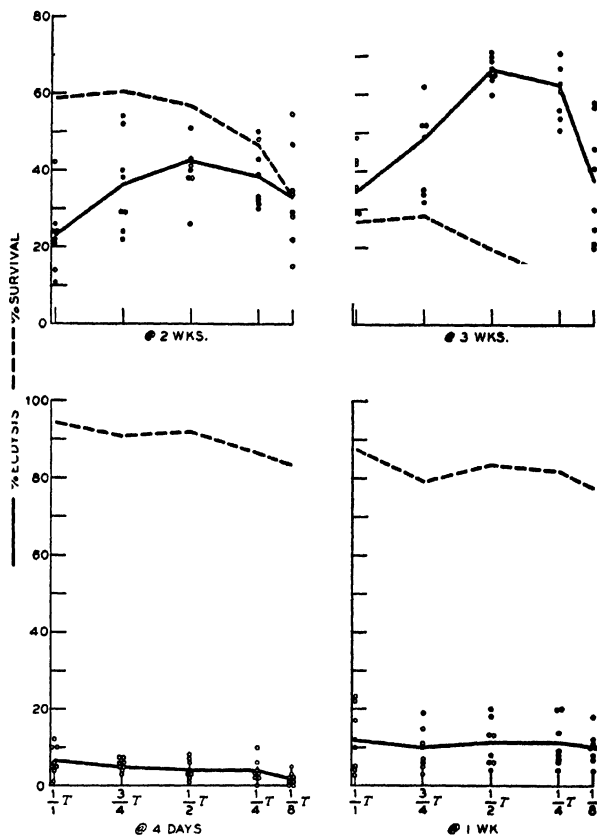
2. Balanced salt solutions at the physiological concentrations isotonic for mammalian or bird tissues offer a distinctly less favorable environment for this parasitic nematode than a range of dilutions. With sodium chloride alone in solution, its marked toxicity is decreased with decreasing concentration.

3. The production of parasitic ecdysis under the conditions outlined indicates that the inclusion of liver extract in the culture media suggested by Glaser and Stoll (1938c) is not necessary to bring about the initial phase of parasitic development culminating in ecdysis, although it may play a rôle. In the present experiment some ecdysis occurred even in distilled water. In such an exceptional environment it may have been made possible by substances set free into the water through the early death of many of the inoculated larvae (or by the trace of tap water in the inoculating drop, 0.075 ml. from the final tap water wash). Ecdysis production without the aid of liver extract is nevertheless a delayed phenomenon. In the experiment with unsealed tubes it had not occurred in one week, had scarcely begun at 2 weeks, and in the most favorable solutions, Tyrode and Ringer, was not very apparent until sometime after the 3rd week.

4. There is no indication that the pH affected the degree of ecdysis. In Exper. 1 the salt solutions were not adjusted, inasmuch as a test of a range of pH requires adding buffer, which tends to interfere with the direct test of the salt solution ingredients alone. The Tyrode was somewhat alkaline (8.0–8.6), Ringer, saline, and water slightly acid (6.2–6.8). It is true that ecdysis occurs in media as acid as pH 3.0 (Glaser and Stoll, 1938c). But the idea has had to be modified that the beginning of parasitic development of *Haemonchus* in vitro requires a strongly acid environment, despite the fact that the abomasum of the sheep on the mucosa of which this stage occurs in nature, is markedly acid (3.5–3.9).

Exper. 2. Various tests have been undertaken to determine whether the good ecdysis rates of the balanced salt solutions of Exper. 1A could not be produced more promptly. Exper. 2 illustrates a successful, reproducible result. In this experiment Tyrode solution alone was used in concentrations

from 1/1 to 1/8, the tubes (100×10 mm.; with 2 ml. solution each) were pre-incubated 24 hours before inoculation with an average of 175 *H. contortus* per tube, and sealed with "Parafilm" before incubation. Graph 2 shows the results with their averages of 160 individual tubes, expressed as the percentage of



GRAPH 2. Ecdysis in sealed tubes of Tyrode solution at dilutions from 1/1 to 1/8, at 4 days, and 1, 2, and 3 weeks. The individual results for each of 8 tubes at each examination show the variation from the mean value, full line. The broken line shows the mean survival rate in the same tubes.

larvae in each tube which had undergone ecdysis at the 4 examination periods, at 4 days and 1, 2, and 3 weeks. The average percentage of surviving larvae at each examination is given as a dash line, the survival rate for individual tubes, which showed about the same amount of variability as the rates of ecdysis, being omitted to simplify the graph.

Under conditions of pre-incubating and then sealing the tubes, there was an acceleration of the ecdysis production as compared to Exper. 1A. It had begun by the 4th day, had doubled in a week, and showed significant increases at 2 and 3 weeks. The beneficial effects of dilutions of the usual Tyrode concentration were not discernible before the 2nd week. By the 3rd week the Tyrode series shows markedly smaller ecdysis values at 1/1 and 1/8, better at 3/4 and 1/4, and in 1/2 Tyrode concentration the 8 tubes gave individual results from 60-71 per cent, with an average of 66. Except that it took between 2 and 3 weeks to reach these ecdysis rates, the mid ranges of the Tyrode dilutions were adjudged to have produced excellent results.

Whether Tyrode is the best balanced salt solution for use in producing parasitic ecdysis is being further investigated. Preliminary observations indicate that the glucose contained in it does not play a significant rôle, for it can either be omitted or increased to quadruple concentration, without modifying the degree of parasitic ecdysis produced. Some tests, the inconsistencies of which have not yet been resolved, suggest the possibility of securing sufficiently good ecdysis with balanced salt solutions alone, perhaps comparable in shortness of time and completeness to the ecdysis values now obtainable with a supplement of liver extract such as illustrated in the next experiment.

Liver Extract

While parasitic development of infective *Haemonchus* larvae can be initiated in their absence, certain liver extracts have been found to accelerate this biological process. Glaser and Coria (1933) cultured *Paramecium caudatum* free from other living microorganisms with the aid of commercial Lilly liver extract No. 343, and also with aqueous extracts made from fresh livers. Trager (1935) successfully cultured mosquito larvae under sterile conditions with the same Lilly extract. On comparative test the effectiveness of various concentrations of Lilly liver extract No. 343 (made from swine livers), in initiating parasitic development of *H. contortus* larvae cannot be distinguished from the effectiveness of Tyrode or Ringer solution alone. With aqueous extracts made in the laboratory from certain fresh livers, better results have been secured. Despite many experiments, with emphasis upon care in processing and standardization of tests, extracts from sheep, lamb, and calf livers showed great variability in potency. The reason for this has not yet been determined. Rabbit liver extract showed some potency, swine liver extract practically none, both derived from animals not natural hosts of *Haemonchus*.

More recently an extract prepared from Difco Bacto-Liver, when used in certain concentrations, has been uniformly successful in producing ecdysis more promptly. This extract can be titrated in terms of the amount of whole liver from which it is derived and has been found to show a similar concentra-

tion of effective substance in different lots purchased, even when the initial stock solutions are first made up at differing concentrations.

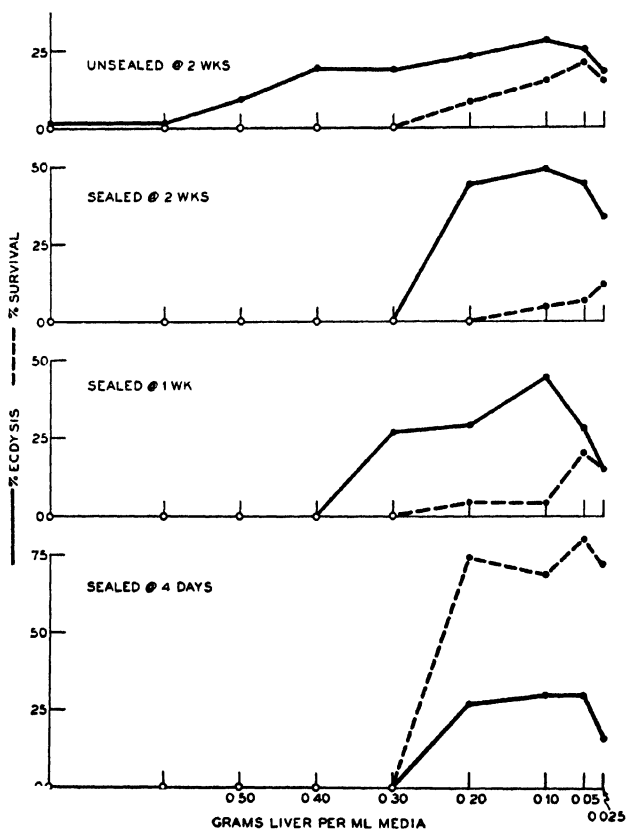
Bacto-Liver is bovine whole liver powder, 27 grams of which are stated to be the equivalent of 100 grams fresh liver. The aqueous extract has been prepared as follows: Distilled water is added gradually to 27 grams of liver powder to make first a paste and then a suspension, employing in all 173 ml. water. If acidity is not as much as pH 5.0, sufficient 3 per cent H_2SO_4 is stirred into it to reach this value. The mixture, in a covered dish, is allowed to infuse for two days in the refrigerator, with occasional stirring. It is then boiled to coagulate the proteins, and these are removed by pressing out through gauze and filtration through paper. The volume is brought to 100 ml. at this point, by further evaporation if necessary. The extract, rich brown in color, is then tubed, autoclaved, and refrigerated until used. If the boiling and protein removal have been inadequate, considerable amounts of fine precipitate will form in the tubes on cooling, the presence of which in experimental tubes causes difficulties in larval examinations. To obtain clear extract the supernate may be passed through a Berkefeld candle, or the extract refiltered through paper and again autoclaved. The making of the extract, as outlined, follows the procedure used for fresh livers, except that for the latter the amount of water employed in the first step (infusion) is equivalent in weight to the ground liver used. The sterile extracts are characteristically of pH 5.0-5.6, with 1 ml. extract representing 1 gm. original liver, and a salt concentration (calculated as NaCl) of about 80 mM/L.

A representative experiment illustrates the early increase in ecdysis produced in the presence of liver extract, and the range of extract concentration found most favorable.

Exper. 3. This was arranged to test the potency of the liver extract in graded amounts from 0.750 to 0.025 gm. original liver per one ml. medium made with a net concentration of $3/4$ Tyrode solution (120 mM/L). The medium at each concentration was adjusted to pH 5.0 under sterile conditions, using N/1 and N/10 HCl. After adjustment the medium was distributed in 2 ml. amounts in 100×10 mm. tubes, and to each of these was then added an average of 80 sterile *Haemonchus* in a small drop of $1/8$ Tyrode solution. "Unsealed" tubes had a cotton plug only; the others were covered in addition with the "Parafilm" seal. The individual results (not averages) of 36 tubes examined after 4, 7, and 14 days incubation at $38\frac{1}{2}^\circ C$ are shown in Graph 3.

In sealed tubes containing extract representing 0.40 grams or more of liver per ml. media, the larvae all died before ecdysis. In such cases, with a concentration of liver extract so great as to be toxic, the dead larvae are stained a deep brown, some nearly black. It is apparent that the optimal concentration of liver extract is in the range 0.20-0.05 gm. per ml. media, a usual finding with extract made as described from Bacto-Liver. One tube at the 7 day ex-

amination showed appreciable ecdysis at 0.30 gm., with all the larvae then dead. Despite such an aberrant tube at the margin of the favorable range, which not infrequently occurs, the results in general are highly consistent. The increased ecdysis at 7 days, over that at 4 days, was accompanied by a similar increase in the death rate, the results at 7 and 14 days not differing



GRAPH 3. Titration of liver extract (grams original liver per ml. media) in production of first parasitic ecdysis of *Haemonchus contortus*.

essentially. Survivors were exclusively larvae which failed to develop. Dead young 4th stages had shown no evidence of growth. This failure to survive is uniformly the case and is considered evidence that liver extract and Tyrode solution do not contain materials utilizable as food for parasitic growth, even though in combination they furnish an environment in which accelerated development into the 4th stage occurs.

The upper part of Graph 3 shows the results of tubes left unsealed, and examined at 2 weeks. Parasitic ecdysis here took place over a wider range of concentration of liver extract. In the four concentrations tested at 0.200 to 0.025 grams liver per ml. medium the unsealed tubes did not reach levels as high (19 per cent less on the average) as similar concentrations in sealed tubes. At 0.75 to 0.40 from a minimum to moderate amounts of ecdysis occurred in unsealed tubes, when, in contrast, it was absent from the sealed, although in both sets at these concentrations all larvae were dead at the end of the 2nd week. The most favorable concentration—0.10 gm. liver per ml. media—was alike in both sealed and unsealed tubes.

There is clearly enhancement of ecdysis here by liver extract, over the results with Tyrode solution alone in either Exper. 1 or 2. Contrasting the unsealed tubes at 2 weeks, Exper. 1A showed the mere beginning of ecdysis with most of the 3rd stage larvae still alive; whereas Exper. 3 showed good ecdysis values, although most of the larvae, whether 3rd or 4th stages, were dead, indicating virtual completion of the test. Contrasting the sealed tubes in Exper. 3, the values with optimal amounts of liver extract are greater at 4 days than Exper. 2 with Tyrode shows in any tube at any dilution in 7 days; by the end of 2 weeks without liver extract there are some values comparable to the values in Exper. 3 at 1 week. The final amount of ecdysis at 2 weeks with liver extract compares approximately with the mean value of $3/4$ Tyrode solution in Exper. 2 at 3 weeks.

As a part of Exper. 3, not shown graphically, a test was made of the same liver extract after re-autoclaving. Using stock extracts at pH 5.0-5.6, different test samples of which had been re-autoclaved successively for 1, 3, 7, and 15 days, full potency was demonstrable. Extract re-autoclaved once at pH 7.0 retained its potency; extract re-autoclaved once at pH 8.0 showed some loss in effectiveness.

Ecdysis Rates in Relation to Tube Size, Amount of Media, and Number of Larvae Inoculated

As already noted there are differences in the rate and amount of parasitic ecdysis produced in sealed and unsealed tubes. This would appear to hinge primarily on the question of oxygen tension, and to the extent that this is true such variables as the size of tube, amount and depth of media, and number of larvae inoculated would all play a rôle in determining the optimum conditions. Experiments 1-3 have already made clear that contact of the media with free air in unsealed tubes represents a less favorable ecdysis-producing environment than when the tubes are sealed. (See also Exper. 4.) Shallower depths of media, devised for either large or small tubes (i.e., differing volume-depth relationships) accentuate the poorer parasitic environment of relatively large air volumes in contact with small volumes of media. In the other direction, a

few cases have occurred in which, using small tubes, sealed, nearly full of media and thus with very little volume of air above the surface, inoculations with large numbers of larvae have resulted in their death, apparently from asphyxiation. At least the tubes on examination at 4 days and later contained only dead larvae and no sign of ecdysis, although free from contamination, and with the same media in other tubes permitting ecdysis.

For most experiments tubes have been employed of 100×10 mm. size, in which 50-200 larvae have been inoculated into 2 ml. media. The tubes have been selected for uniformity so that the resulting depth of media is about 35 mm. If such tubes with 2 ml. media are contrasted for ecdysis with 3 ml. media, no clear difference in results with similar numbers of larvae has been demonstrable. Thus in an experiment with Tyrode-liver extract solution it was possible to contrast the degree of ecdysis production in 54 tubes with 2

TABLE I

Ecdysis Production with H. contortus, Using Graded Numbers of Larvae in Sealed 100×10 Mm. Tubes, with 0.1 Liver Extract-3/4 Tyrode Media, pH 5.0, in 2 and 4 Ml. Amounts, Producing Depths of 35 and 70 Mm. Individual Tube Results at 4 Days

| Set A. 2 ml. media, 35 mm. depth | | | Set B. 4 ml. media, 70 mm. depth | | |
|---------------------------------------|----------|--------------|---------------------------------------|----------|--------------|
| Number of completed ecdyses (sheaths) | Ecdyses | Larvae alive | Number of completed ecdyses (sheaths) | Ecdyses | Larvae alive |
| | per cent | per cent | | per cent | per cent |
| 26 | 27 | 92 | 6 | 21 | 83 |
| 179 | 37 | 72 | 94 | 16 | 72 |
| 466 | 53 | 93 | 298 | 30 | 78 |
| 1451 | 51 | 84 | 975 | 44 | 82 |

ml. against 43 with 3 ml.; average ecdysis rates in the former were 23.3 per cent, in the latter 22.7 per cent, evidencing no difference in this range.

Exper. 4. When 2 ml. and 4 ml. amounts are contrasted with varying numbers of larvae, the rôle of the numbers of *Haemonchus* introduced and the interrelationships of the media volume and air volume in the sealed tubes are involved. In *Exper. 4*, a 0.1 gm. liver extract-3/4 Tyrode medium at pH 5.0 was used, with special care in selection of tubes so that 2 ml. in Set A reached a depth of 35 mm., 4 ml. in Set B, 70 mm. They were inoculated in parallel with graded number of larvae per tube, from less than 100 to over 2000. The results of examinations at 4 days are shown in Table I. In both the A and B sets there is in general increasing degree of ecdysis with increased numbers of larvae. However, in inoculations of the same general size there is increased ecdysis in the 2 ml. as contrasted with the 4 ml. amounts of media.

Exper. 5. A variety of experiments has been undertaken to explore these relationships, which are still unsatisfactorily understood. A synopsis of one such, *Exper. 5*, is given, in which 0.1 gm. liver extract-3/4 Tyrode medium at

pH 5.0 was used. In this experiment 100×10 mm. tubes (total volume $5\frac{1}{2}$ ml.) with from $\frac{1}{2}$ to 4 ml. medium, were compared with 100×13 mm. tubes (total volume 10 ml.) with from 1 to 7 ml. medium, and with 150×13 mm. tubes (total volume 14 ml.) with from 1 to 12 ml. medium. Half were inoculated with an average of 105 larvae, a parallel series with 390 larvae per tube, for examination at 4, 7, and 14 days. Due to some sealed tubes developing contaminations, 93 were eventually examined, classifiable in 33 different relationships of tube size, media volume, and size of inoculation. A control set of tubes (in which 24 of these relationships were paralleled) was left unsealed and examined at 14 days.

The differing amounts of medium used in various tubes gave cross-checks on comparable depths with dissimilar volumes, and vice versa, in relation to two densities of *Haemonchus* larval population. In general throughout the

TABLE II

Ecdysis Rates (Mean of Values at 4, 7, and 14 Days) in Relation to Available Oxygen per 100 H. contortus, Comparing Tubes with Differing Ratios of Larvae and Amount of Media in Tubes of Different Sizes. See Text

| Total ml. oxygen available per 100 <i>H. contortus</i> | Per cent ecdysis | | | | | | | |
|--------------------------------------------------------------|------------------|-----|-------|-------|-------|-------|-------|-------|
| | 1-4 | 5-9 | 10-14 | 15-19 | 20-24 | 25-29 | 30-34 | 35-39 |
| .05-.24 | | | 1 | 2 | 1 | | 1 | 1 |
| .25-.49 | 1 | 5 | 1 | 1 | 2 | | | |
| .50-.74 | 1 | 1 | 1 | 1 | | | | |
| .75-.99 | 1 | 1 | 3 | | | | | |
| 1.00 and over | 1 | 6 | | 1 | | | | |
| Unsealed | 4 | 9 | 1 | | | | | |

series inoculations with 390 larvae provided better percentages of ecdysis than with 105. There was a lag in, as well as a decreased total, ecdysis in tubes with small amounts of medium. Less ecdysis occurred in the large tubes than in either of the smaller sizes. The unsealed tubes in each set gave inferior results compared to the sealed.

In view of the planned diversity of conditions in the experiment, it was analyzed from the standpoint of the total amount of free oxygen available to the larvae, computed for the actual count of larvae in each tube. The mean ecdysis rates of the three examinations for each tube size with a given amount of medium are shown in Table II in relation to the amount of oxygen sealed in the tube per 100 *Haemonchus*. The oxygen computations were made assuming the usual amount in air, at standard pressure, with solubility of the oxygen in the medium comparable to that in water at the same temperature; "ml. total oxygen" was derived from the sum of the dissolved oxygen plus that in the enclosed air of the sealed tube.

Such an analysis is admittedly an approximation, but does to a certain extent define, for the first parasitic ecdysis of *Haemonchus*, the combined rôle of media volume, total oxygen volume dissolved in medium and present in the air in the sealed tube, and number of larvae introduced. It is immediately apparent that the best average ecdysis rates occur exclusively in the low values of oxygen available per 100 larvae. Reciprocally, the poorest ecdysis rates are missing from that same oxygen level, but are especially numerous in the highest oxygen level, classified here as 1.00 ml. oxygen or more per 100 larvae. In complete support of the latter grouping is that of the unsealed controls, the media of which were, of course, uninterruptedly in contact with free air, and which show a similar clumping of low average ecdysis rates.

This expression of the favorable ecdysis-producing environment is not peculiar to Exper. 5. As illustration it may be pointed out that the five highest ecdysis rates in Exper. 4, Table I, are in tubes whose computed oxygen values fall in the line of the smallest amount oxygen per 100 larvae of Table II.

These general relationships suggest that for best results in the production of ecdysis with *Haemonchus* a certain low oxygen tension is desirable, and that this is reached rather more readily in small test tubes, with moderate amounts of media, sealed after inoculation. As a practical procedure in comparing ingredients and culture conditions, larval inoculations of about 100 *Haemonchus* per 100 \times 10 mm. tube containing 2-3 ml. medium have been favored because they are more serviceable for intra-experimental comparisons with a saving of time in making examinations, than tubes containing large numbers of organisms. Such favored tube sizes, etc., have oxygen values which strike the middle of those specified in Table II.

DISCUSSION

The experiments here reported show that the first parasitic ecdysis of *H. contortus* may be accomplished in vitro under sterile conditions with certain definite expectations. It succeeds with balanced salt solutions, alone, such as Tyrode or Ringer, and essentially fails with unbalanced sodium chloride solution. It succeeds better with balanced salt solutions at less than the usual concentrations which are physiological for mammalian or bird tissue. The optimal range of dilution is from about 1/4 to 3/4 representing a concentration of 40 to 120 millimols per liter. It succeeds better, under the conditions of the experiments here reported, in small tubes with moderate amounts of media, and when the tubes are sealed rather than unsealed; and better with larger than smaller numbers of larvae.

This first parasitic ecdysis, while it is accomplished in balanced salt solutions alone, is accelerated with certain concentrations of an aqueous liver extract, of which the most reliable to date has been prepared from Difco Bacto-Liver.

This production of parasitic ecdysis in *Haemonchus* is a biological phenomenon, and not an extension of the chemical exsheathing process by which the larvae have been earlier sterilized. The exsheathing of the infective larva is of a larval stage with a sheath already formed and ready to cast. Parasitic ecdysis is the culmination of a "pupation," involving the formation of the new sheath at the end of a process in which morphological changes occur in the mouth parts of the larva, interpreted functionally as making it able to attack the mucosa of the ruminant 4th stomach, its normal residence. The development ending in parasitic ecdysis does not occur in larvae kept under refrigerated conditions in the same solutions in which they will undergo the process when incubated at 38½°C. So far it has not been observed at room temperature, although tests have not been exhaustive on this point. Contrary to expectation, it has not been dependent upon either acid or alkaline environments, taking place in both. It occurs better under conditions of restricted oxygen tension.

From these facts it would appear that parasitism for *H. contortus* begins when there has been substituted for the free-living environment outside the host, one in which there is a physical environment including lessened oxygen tension and a continuously higher temperature, comparable to that of the sheep abomasum, together with a certain chemical balance in the menstruum in which it finds itself comparable to that reproduced by a dilute balanced salt solution. The further fact that parasitic ecdysis as a process is accelerated in the presence of aqueous liver extract suggests that under ordinary parasitic conditions certain biological factors stimulate the reaction. The substance or substances in liver extract may play this rôle in nature, or may constitute an *in vitro* substitute.

It is significant that *H. contortus* completes this first parasitic ecdysis without dependence on food other than reserves stored in its own cells and then, as a young 4th stage larva, dies, if appropriate food is not available. Possibly this is a characteristic of parasites which under natural conditions reach a new biological stage of development promptly after reaching a new host. The evidence is not clear in Ferguson's (1940) culture of a metacercaria which evolves promptly to an egg-producing trematode whether the media constituents, which, besides dilute Tyrode, include serum and yeast extract, are of actual nutritive assistance to the parasite; *P. minimum* like *H. contortus* completes a first parasitic "stage" in the host within less than 48 hours.

Once the fact is demonstrated that salt concentrations which are appreciably less than those isotonic for tissues prove best as a parasitic environment for *H. contortus* larvae, it is seen to be clearly related to *Haemonchus* biology. This parasite, in nature living superficially on the wall of the ruminant stomach at this stage, is to be thought of as inhabiting the lumen rather than the tissues,

even though it lies very closely in apposition to the epithelial cells. Gastro-intestinal helminths in general may require such a diluted salt solution in vitro, and Ferguson (1940) has recently indicated that for the strigeid, *P. minimum*, a 5/8 Tyrode solution was the most favorable concentration. Cobb (1924) "raised the question as to the best solution strengths to keep nemas alive, noting that he had found normal saline unsatisfactory for free living nemas," but whether this was due to toxicity of sodium chloride or hypertonicity of the solution is not clear. Fenwick (1939) testing the longevity of *Ascaris suum* larvae for saline requirements found they lived longest, 5½ days, in a suitably antagonized saline whose constituents represent full strength Tyrode solution, minus the buffers and glucose (Parker, 1938), with a computed millimolar concentration of 142, in contrast to the 40-120 range found more suitable for *Haemonchus*. This difference may signify that the newly emerged *A. suum* larva requires a medium isotonic with tissue fluids, which would be in harmony with the well known tissue wandering habits of *Ascaris* at this stage in its life cycle.

Of interest in this same connection is the circumstance that conditions favoring the first parasitic ecdysis of *H. contortus* in vitro are not necessarily effective for other forms. Tests with *Trichostrongylus axei* show that it does not respond as does *Haemonchus*, although the infective larvae are readily exsheathed and sterilized for test.

SUMMARY

The first parasitic ecdysis (end of 3rd larval stage) of *Haemonchus contortus* may be secured in vitro with balanced salt solutions such as Ringer and Tyrode. (Sodium chloride alone is toxic.) Better results are obtained with less than the usual physiological concentrations for tissues, the favorable range being 1/4 to 3/4 (40-120 mM/L). In incubated unsealed tubes ecdysis begins after a week, reaches its best values after a month. In sealed tubes the time is approximately halved. With the supplement of a small amount of liver extract (aqueous), ecdysis proceeds more promptly, and under appropriate conditions may occur with from a third to a half of the worms in the culture tubes in 4 days. The liver extract is titratable in terms of the amount of original liver represented per ml. of culture media. Better ecdysis rates also occur under conditions of restricted oxygen, as secured in small tubes with moderate amounts of media, pre-incubated before inoculation, and then sealed.

Distinction between factors favoring the first parasitic ecdysis in vitro and those which allow development beyond it, is possible because of the dependence of the larvae on endogenous food reserves during this initial step in parasitic life.

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HETEROLOGOUS TRANSPLANTATION OF MAMMALIAN TUMORS

I. THE TRANSFER OF RABBIT TUMORS TO ALIEN SPECIES

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PLATES 22 AND 23

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The demonstration of the transplantability of cancers gave early impetus to the conception of autonomy and led immediately to the hope that their independent nature would eliminate species barriers and allow growth in alien hosts. Numerous experiments were performed with this point in view, but the majority of workers have reported failure or results of a controversial nature, so that at the present time, the general concept of cancer autonomy remains restricted in scope and has not been extended to include growth in a foreign species.

The extensive literature dealing with the heterologous transplantation of mammalian tumors has recently been reviewed and reference to the many experiments will not be repeated here (1). Successful transfer between species was undoubtedly effected in several instances. Murphy transferred the Jensen rat sarcoma to the developing chick embryo and was able to carry the tumor serially in this host (2). Transfer back to the rat resulted in takes but all attempts to transplant the tumor to adult chickens failed. Shirai reported briefly the transplantation of a rat sarcoma to the brains of adult mice but, apparently, serial transfers were not attempted (3). Putnoky transplanted Ehrlich's mouse carcinoma subcutaneously to adult rats (4) and has maintained the tumor by serial transfer in this species since 1929. A large inoculum of 300 to 500 mg. of tumor is used and growth is rapid. Regression begins by the 10th day and transfer must be made at this time. Histologically, the appearance of the tumor is identical with that in the mouse host. Finally, Lucké and Schlumberger have recently reported the successful transfer of a frog tumor to other cold blooded species (5).

Other claims of successful heterologous transfer cannot be accepted without reservation. Keysser and his associate, Hegner, have reported the successful transfer of carcinomata and sarcomata from the mouse to the vitreous humor of rats but their claim could not be substantiated by Woglom (6-9). These authors have also described the heterologous transplantation of human cancers, using the same technique. However, they were unable to rule out the possibility that the tumors observed in the experimental animals were of spontaneous origin and, because of the time relations and the morphology of the growths, this seems highly probable. Such a view was held by Aschoff

and is shared by others who have reviewed the experiments critically (10). Smirnova recently described the successful heterologous transplantation of a variety of tumors to the anterior chamber of the eye but the account is not sufficiently detailed to allow critical judgment (11).

Experience with the homologous transfer of rabbit tumors in this laboratory had demonstrated the advantages of the anterior chamber of the eye as a transplantation site. The ease with which transfer was effected, utilizing this mode of implantation in contrast to the complete failure encountered in other bodily regions, suggested that it might prove an efficient means of transferring tumors between animals of different species. This suggestion was subjected to test, and the results of early experiments were recorded in a preliminary report in 1938 (12). The success which attended the experiments led to broader investigations and the work has been extended in various directions. Other rabbit and human tumors have been utilized and studies have been undertaken in an attempt to discover the factors determining transplantability to an alien species. In addition, the specific nature of the tissue grown in the foreign host and the relation of alterations in the tissue to autonomic properties have been investigated.

The object of the present paper is to report in detail the results of the heterologous transplantation of a series of rabbit tumors. A second paper will be concerned with the successful transplantation of human tumors and a discussion of the results of the two groups of experiments. Subsequent reports will deal with additional investigations indicated above.

Material and Methods

The tumors used in this series of experiments consisted entirely of rabbit carcinomata and included the uterine cancer, H-31 (13), the breast cancers, T-36 and B-240 (14), and the Brown-Pearce tumor (15). Control experiments were performed with normal adult endometrial and mammary tissue as well as with embryonic parts. The foreign species tested were guinea pigs, swine, goats, and sheep.

The animals were not subjected to special treatment either before or after transfer but were maintained under ordinary conditions of cage or pasture life. The tumors were always carried for one or more generations in the anterior chambers of rabbits' eyes before heterologous transfer was attempted. On the other hand, normal tissues were used directly on removal from the primary host.

The technique of transplantation employed has been described already (13), but because of the significance of the mode of inoculation in the present connection, the essential features will be repeated. The eye to be used is first anesthetized with a 5 per cent solution of cocaine. The anterior chamber is opened close to the superior margin of the corneoscleral junction by means of a short quick stab with a double-edged corneal knife. A small amount of aqueous humor is allowed to escape, and a fragment of tumor tissue measuring approximately one millimeter in diameter is inserted into the chamber,

using either forceps with fine serrated points or a small beveled trocar. The fragment is then forced into the inferior angle of the iris by applying light pressure along the corneal surface with a blunt instrument. The corneal incision closes after withdrawal of the inserting instrument and further care is unnecessary.

H-31 Tumor

The first attempts to transfer rabbit tumors to an alien species were made with the adenocarcinoma, H-31. This tumor originated in the endometrium of a rabbit's uterus, and its growth characteristics following homoplastic transplantation in the anterior chamber of the eye have been extensively studied (16). In brief, takes occur in 90 per cent of rabbits, growth of the fragments is evident during the 2nd week after transfer, and the chamber is usually completely filled by the 30th day. Regression of the tumor follows in approximately 70 per cent of cases, but in others growth is progressive and metastasis occurs after 6 to 8 months.

Guinea pigs, swine, and goats have been employed in heterologous transplantation experiments, and in each instance transfer of the tumor to the new species has been successfully performed.

Guinea Pigs.—The first experiment utilizing guinea pigs was undertaken in February, 1938. Tumor fragments derived from the eye of a rabbit of the sixth serial generation were placed in the anterior chambers of two 300 gm. pigs. Growth of the fragments was evident on the 16th day and in both instances the tumor had increased to fill the chamber by the 50th day.

Other successful transfers of this tumor from the rabbit host to guinea pigs were made in March, August, and November of the same year and in February of the following year (Table I). The material employed in these instances was obtained from the 13th and 15th anterior chamber generations and the first and sixth testicular generations in the rabbit series. It should be emphasized, however, that subsequent attempts to transplant tumor tissue derived from later eye generations in the rabbit were unsuccessful and transfers performed in October, 1939, and in January and April, 1940, using tissue from the 26th, 29th, and 32nd generations, gave rise to no takes.

Serial transplantation of the growths resulting in guinea pigs was successfully effected. The tumor arising from the first experiment performed in February, 1938, was carried by consecutive anterior chamber transfer through four guinea pig generations, while that resulting from the November experiment of the same year was carried for five serial generations. Serial transfers were discontinued in both cases for reasons of economy and there is no cause to believe that the tumors could not have been perpetuated indefinitely by continued serial passage.

Control inoculations of whole tumor fragments or of cellular emulsions into the testicles, muscles, and subcutaneous tissues of other guinea pigs were performed throughout the series of experiments. Tumor tissue derived from guinea pig generations as well as from rabbit hosts was used, but no takes occurred.

As a rule, the presence or absence of takes in the guinea pig's eye could be determined during the 2nd week after transfer, and at this period, a slight increase in size and a

pinkish coloration distinguished growing fragments. Occasionally, however, the transplants remained unchanged in appearance for as long as 110 days but eventually grew to fill the chamber. Vascularization was usually apparent by the 3rd week, but in instances such as the above, the fragments received no visible blood supply during the latent period.

In one instance the chamber was completely filled with tumor on the 15th day, but as a rule, growth proceeded at a slower rate than in the rabbit and 40 to 50 days were generally required before such a size was attained. Thereafter areas of degeneration appeared and the tumors underwent regression. However, regression was rarely com-

TABLE I

The Results of Transplantation of the Rabbit Carcinoma, H-31, into the Anterior Chamber of the Eyes of Guinea Pigs

| Series No. | Source and generation of rabbit tumor | Date of transfer | Guinea pigs | | Total takes |
|------------|---------------------------------------|------------------|----------------|-------------|-----------------|
| | | | Generation No. | Number used | |
| 1 | Eye, 6th | 1938 | | | <i>per cent</i> |
| | | Feb. | 1 | 2 | 100 |
| | | Apr. | 2 | 5 | 60 |
| | | June | 3 | 6 | 33.3 |
| | | July | 3 | 5 | 20 |
| | | Oct. | 4 | 7 | 42.8 |
| 2 | Testicle, 1st | Mar. | 1 | 5 | 80 |
| 3 | " 6th | Aug. | 1 | 4 | 50 |
| 4 | Eye, 13th | Nov. | 1 | 4 | 100 |
| | | 1939 | | | |
| | | Jan. | 2 | 5 | 60 |
| | | Feb. | 3 | 3 | 66.6 |
| | | Apr. | 4 | 7 | 85.7 |
| | | June | 5 | 5 | 40 |
| 5 | " 15th | Feb. | 1 | 7 | 42.8 |
| 6 | " 26th | Oct. | 1 | 6 | 0 |
| | | 1940 | | | |
| 7 | " 29th | Jan. | 1 | 5 | 0 |
| 8 | " 32nd | Apr. | 1 | 8 | 0 |

plete. On the contrary, small nodules of healthy living tissue persisted and by continued growth again filled the chamber.

Some of the animals were killed for histological examination or to obtain tissue for serial transfer, but the majority were held for continued observation.

Growth was occasionally extremely slow and its course extended over a long period of time. In five instances, the tumor persisted for 500 days without filling the chamber, while in one case growth continued until the animal was killed on the 785th day after transfer and at the end of this period the transplant measured only 2.5 mm. in diameter. However, the tumor was living and the innate growth capacity of its cells unimpaired as was shown by transfer of fragments back to the eyes of rabbits where growth occurred and filled the chambers in 50 days.

A protracted course of growth resulted in other animals from a combination of a slow rate of increase and the persistence of areas of living tissue after regression of the main tumor mass. The following protocol is reproduced in condensed form to illustrate such an occurrence.

June 24, 1938—transfer. Fragment measures 1 mm. in diameter. July 8—pinkish coloration but no increase in size. Aug. 30—no increase in size. Nov. 1—fragment measures 1.5 mm. in diameter. Dec. 23—2.5 mm. in diameter. Feb. 6, 1939—5 mm. in diameter. Mar. 1—1 cm. in diameter. Chamber filled. Mar. 23—beginning regression. Apr. 1—complete regression with the exception of one small living area. Apr. 20—living tumor measures 2.5 mm. in diameter. May 15—5 mm. in diameter. Aug. 15—chamber filled. Sept. 5—regression with persistence of living nodule 1.5 mm. in diameter. Nov. 10—2.5 mm. in diameter. Jan. 15, 1940—animal killed. Living tumor measures 3 mm. in diameter.

At autopsy, the tumors appeared as semi-translucent, pinkish-gray masses. The greater part of the iris was often replaced by the tumor tissue of older transplants, but young, fast-growing tumors were attached over only a small portion of their circumference and, on removal of the cornea, resembled sessile polyps. The cornea was never invaded and the growths never extended into the posterior chamber.

Microscopically, the tumors resembled those resulting from homologous transfers. The epithelial elements were identical in appearance and manifested the same tendency to grow in small acinar bundles or in large, solid, cellular masses (Figs. 1 and 2). The stroma was abundant, well vascularized, and healthy in appearance. Nowhere were there lymphocytic infiltrations or cellular evidences of a foreign body reaction. In very old tumors, epithelial elements were scanty and appeared as single cells or isolated acini embedded in a dense hyaline stroma (Figs. 3 and 4). However, they showed no evidence of degeneration, and successful transfer back to rabbits proved their viability (Fig. 5).

Serial sections were obtained from many organs, and a thorough search was made for metastases. Foci of cells resembling the essential tumor elements in structure and similar to those frequently present in rabbits with other larger metastatic growths were observed in the lymph nodes and lungs in several cases, but in no instance was a lesion found which could be unequivocally identified as a metastasis.

Swine.—The tumor was successfully transferred to the eyes of swine in June and October, 1938, using material derived from the 8th and 12th anterior chamber series in the rabbit, respectively. Two hogs weighing approximately 45 pounds each were used in each transfer. A take occurred in a single animal in the first experiment, but growth resulted in both of the hogs used in October.

A biopsy was performed on the eye of one of the latter animals 15 days after transfer and the greater part of the tumor removed. Fragments of this growth were transplanted to the eyes of two additional hogs and a cellular emulsion inoculated intratesticularly into four others. Growth occurred in the anterior chamber in both instances, but no takes resulted from the testicular inoculation.

Takes were evident in hogs' eyes at the end of a week. Subsequent growth was exceptionally rapid, and in all five animals, the anterior chamber was completely filled by the 15th day. In contrast, fragments of the tumor transferred at the same time to the eyes of control rabbits showed only presumptive evidence of growth on the 15th day.

The ultimate fate of the tumor in this species has not been determined. Complete

regression followed the filling of the chamber in two instances, while in two others small areas of living tissue were present at autopsy, one month after transfer. The tumor apparently had been entirely removed in the remaining animal, for no further growth occurred after biopsy.

Microscopically, the tumor appeared more active than in either the guinea pig or the rabbit (Fig. 6). Epithelial cells were more abundant and tended to grow in solid sheets with less attempt at acinar formation. Connective tissue stroma was scanty, but the tumor was permeated with small, thin-walled blood vessels. A thorough microscopic search was made, but no metastatic foci were found.

Goats.—The tumor was also successfully transferred to the eye of a goat in June, 1938, using material from the eighth rabbit eye generation. Two animals were used in this experiment and a take occurred in one. Serial transfers were not attempted.

Growth was evident in the one instance at the end of 3 weeks. Subsequent progress of the tumor was extremely slow, and after 6 months the fragment, although well vascularized, had no more than tripled in diameter. No further increase in size was noted, and at the end of 10 months regression began. One year after transfer the tumor had completely disappeared, and only a small scar on the iris marked the site of growth.

T-36 Tumor

Successful heterologous transplantation of the tumor, T-36, was effected in guinea pigs, swine, and sheep. The original tumor tissue was obtained from a splenic metastasis of an acinar type breast carcinoma which, at the present time, has been carried by serial anterior chamber transfer through more than 50 generations of rabbits (17). Growth occurs in 100 per cent of rabbits, irrespective of age, sex, or breed. Takes are usually evident on the 5th day and the chamber is often completely filled by the 15th day. Thereafter, regression invariably occurs, and in no instance to date have metastases been found or has it been possible to transfer the tumor to any other site in the body.

Guinea Pigs.—The results of transfer of this tumor to the anterior chambers of guinea pigs are shown in Table II. Successful transplantation from the rabbit host was performed in October and December, 1938, and in March, 1940, using material derived from the first, fifth, and 40th anterior chamber generations. The two latter transfers were discontinued after the first generation, but serial transplantation was performed in the first instance and the tumor has now been carried for 2 years or 48 consecutive generations in the alien species.

Both young pigs weighing as little as 250 gm. and older animals weighing up to a kilo were used in the serial transplantation experiments. The percentage of takes and course of the tumors were similar in both cases, but the growth rate was slower in the older animals and takes could rarely be recognized before the 10th day. On the other hand, growth was almost invariably evident by the 5th day in young pigs, and subsequent progress was rapid. Occasionally, growth ceased and regression occurred before the chamber was filled, and animals of this type were found to be refractory to reinoculation. In the majority of cases, however, growth continued until the chamber was filled. This

usually occurred by the 10th day in young animals, while in older pigs 2 to 3 weeks were required. Further increase in size was attended with bulging of the cornea and degenerative changes in the tumor, but in general, the tissue remained viable and could be transplanted for as long as a week after filling the chamber. Occasionally, the cornea ruptured in consequence of the increased pressure and the tumor protruded externally. Infection and spontaneous regression invariably occurred in such cases.

Growth was progressive and continued throughout the life of the pig in 5 per cent of cases. This is a special feature in the guinea pig and has never been observed in the rabbit despite the fact that the course of the tumor has been followed in more than 400 animals. It occurred almost exclusively in pigs inoculated in February and March and apparently bore no relationship to the status of the animal or the rate of growth.

In such cases, external extension was a gradual process and occurred after a period during which the intraocular growth assumed the shape of a cone with its apex invading a localized corneal area. Perforation began at the apex of the cone and the area widened to form a carcinomatous ulcer. Eventually, the cornea was destroyed and the tumor protruded as a fungating mass.

The majority of animals of this type were killed, but a number were held to determine the eventual fate of the tumor. The course of growth, in such instances, averaged 90 days but in one animal extended for 154 days, and in all cases, terminated in death from pneumonia. At autopsy, the tumors were found to have extended laterally under the conjunctiva and posteriorly into the vitreous humor. The posterior chamber was often completely filled and irregularly distended with tumor which destroyed the retina and choroid but did not invade the sclera. The regional lymph nodes were enlarged but no metastases were visible throughout the body.

Attempts were made in three cases to transplant tissue obtained at biopsy from the fungating eye tumor to other sites in the body including testicle, muscle, and skin. Such a procedure has been successfully used in rabbits when transfer to new sites in normal animals failed, but growth did not occur in the pigs in question. Numerous attempts to transplant the tumor from the eye of one pig to new sites in another were likewise unsuccessful.

At intervals throughout the experiments tumor tissue from guinea pigs was transplanted back into rabbits. Takes occurred in all instances, and the characteristics of the resulting tumors were in no way different from those observed in serial rabbit transfers.

Microscopically, the epithelial elements of the tumor in the guinea pig are identical with those observed in the rabbit, but in contrast to the almost complete structural disorganization found in the latter species, the cells tend to arrange themselves in more or less definite architectural patterns. Transplants of this tumor in the rabbit consist of solid undifferentiated cellular masses, whereas in the guinea pig growth occurs in the form of well defined acinar bundles (Figs. 7 and 8). Curiously, this structural arrangement is almost identical with that observed in the primary breast tumor in the spontaneous rabbit host, while the architecture of transplants in rabbits' eyes bears a close resemblance to that of metastases of the primary breast growth. However, in old, progressive guinea pig tumors the characteristic acinar pattern is lost and replaced by an arrangement of cells in solid sheets and irregular infiltrating cords (Fig. 9). The tumors are supplied with an abundant, loose, connective tissue stroma which carries numerous thin-walled blood vessels.

TABLE II

The Results of Transplantation of the Rabbit Carcinoma, T-36, into the Anterior Chamber of the Eyes of Guinea Pigs

| Series No. | Source and generation of rabbit tumor | Date of transfer | Guinea pigs | | Total takes |
|------------|---------------------------------------|------------------|----------------|-------------|-----------------|
| | | | Generation No. | Number used | |
| 1 | Eye, 1st | 1938 | | | <i>per cent</i> |
| | | Oct. | 1 | 2 | 50 |
| | | " | 2 | 6 | 50 |
| | | Nov. | 3 | 8 | 37.5 |
| | | " | 4 | 6 | 16.6 |
| | | 1939 | | | |
| | | Jan. | 5 | 5 | 20 |
| | | Feb. | 6 | 5 | 60 |
| | | Mar. | 7 | 5 | 100 |
| | | " | 8 | 6 | 100 |
| | | Apr. | 9 | 5 | 100 |
| | | " | 10 | 6 | 100 |
| | | " | 11 | 5 | 20 |
| | | May | 12 | 6 | 66.6 |
| | | " | 13 | 6 | 83.3 |
| | | " | 14 | 6 | 83.3 |
| | | June | 15 | 5 | 100 |
| | | " | 16 | 6 | 100 |
| | | July | 17 | 6 | 83.3 |
| | | " | 18 | 6 | 83.3 |
| | | " | 19 | 6 | 83.3 |
| | | Aug. | 20 | 6 | 66.6 |
| | | " | 21 | 6 | 100 |
| | | Sept. | 22 | 6 | 100 |
| | | " | 23 | 6 | 66.6 |
| | | Oct. | 24 | 6 | 66.6 |
| | | " | 25 | 5 | 100 |
| | | " | 26 | 6 | 83.3 |
| | | Nov. | 27 | 6 | 100 |
| | | " | 28 | 5 | 100 |
| | | " | 29 | 4 | 100 |
| | | Dec. | 30 | 13 | 100 |
| | | 1940 | | | |
| | | Jan. | 31 | 6 | 83.3 |
| | | Feb. | 32 | 6 | 100 |
| | | " | 33 | 12 | 75 |
| | | Mar. | 34 | 8 | 100 |
| | | Apr. | 35 | 7 | 100 |
| | | " | 36 | 20 | 85 |
| | | May | 37 | 15 | 93.3 |
| | | " | 38 | 14 | 92.8 |
| | | June | 39 | 6 | 100 |
| | | " | 40 | 6 | 100 |
| | | " | 41 | 6 | 83.3 |

TABLE II—*Concluded*

| Series No. | Source and generation of rabbit tumor | Date of transfer | Guinea pigs | | Total takes |
|--------------------|---------------------------------------|------------------|----------------|-------------|-----------------|
| | | | Generation No. | Number used | |
| 1 (<i>cont.</i>) | Eye, 1st | 1940 | | | <i>per cent</i> |
| | | July | 42 | 6 | 66.6 |
| | | " | 43 | 6 | 66.6 |
| | | Aug. | 44 | 6 | 66.6 |
| | | " | 45 | 5 | 80 |
| | | " | 46 | 5 | 100 |
| | | Sept. | 47 | 14 | 100 |
| | | " | 48 | 8 | 100 |
| 2 | Eye, 5th | 1938 | | | |
| | | Dec. | 1 | 4 | 100 |
| 3 | Eye, 40th | 1940 | | | |
| | | Mar. | 1 | 10 | 100 |

The iris was infiltrated with tumor cells in all cases and the ciliary body was almost invariably invaded. In old growths, extensions could be traced under the palpebral conjunctiva, but the muscle of the eyelid was never involved. All organs of the body were carefully searched but metastases were not found.

Swine.—The tumor was successfully transferred to hogs in February, 1939, and again in August, 1940, with tissue derived from the 10th and 51st rabbit generations (Fig. 10). Two animals were used in each experiment and takes occurred in all instances. Serial transplantation to a second generation was undertaken as part of the first experiment and here also growth occurred in all of the animals used.

Evidences of growth were observed by the 7th day in all cases. The chambers of all the animals concerned in the first experiment were filled by the 23rd day, but then regression occurred and a month later no living tissue remained. On the other hand, regression occurred 2 weeks after transfer when the chamber was only one-fourth filled in both animals of the second experiment.

Microscopically, the cells were somewhat smaller and contained less cytoplasm than those found in rabbit transplants, but in other respects the growths in the two species were identical.

Sheep.—In March, 1939, tumor tissue derived from the 8th anterior chamber generation in the rabbit was transferred to the eye of a sheep. Growth was apparent at the end of a week and the chamber was filled with tumor after 20 days. Regression then occurred, and the growth was completely necrotic when the animal was killed at the end of one month.

B-240 Tumor

The papillary type rabbit breast carcinoma, B-240, was successfully transplanted to the eyes of guinea pigs, but transfer to other alien species has not been attempted. Experiments were performed with material originally derived from the primary mammary growth and from a lung metastasis in the spontaneous rabbit host. Under transplantation in

homologous species, growth of the tumor occurs in approximately 60 per cent of males and 37 per cent of females (17). Takes may be recognized by the 15th day and the chamber is filled between the 35th and 50th days. Regression usually follows, but progressive growth occurs in a small number of cases. Metastases have never been found.

Guinea Pigs.—The results of transfer of this tumor to the anterior chambers of guinea pigs are recorded in Table III. Equal numbers of male and female pigs were used in the series of experiments, and it is significant that both of the takes occurred in males.

TABLE III

The Results of Transplantation of the Rabbit Carcinoma, B-240, into the Anterior Chamber of the Eyes of Guinea Pigs

| Series No. | Source and generation of rabbit tumor | Date of transfer | Guinea pigs | | Total takes |
|------------|---------------------------------------|------------------|----------------|-------------|-----------------|
| | | | Generation No. | Number used | |
| | | <i>1938</i> | | | <i>per cent</i> |
| 1 | P.* Eye, 2nd | June | 1 | 4 | 25 |
| | | July | 2 | 5 | 0 |
| 2 | M.† Eye, 1st | Aug. | 1 | 4 | 0 |
| 3 | M. Eye, 4th | Sept. | 1 | 6 | 0 |
| 4 | P. Eye, 5th | Oct. | 1 | 4 | 0 |
| | | <i>1939</i> | | | |
| 5 | P. Eye, 6th | Jan. | 1 | 5 | 0 |
| 6 | P. Eye, 7th | Feb. | 1 | 5 | 0 |
| 7 | M. Eye, 8th | June | 1 | 8 | 12.5 |
| | | Sept. | 2 | 6 | 0 |
| | | <i>1940</i> | | | |
| 8 | M. Eye, 12th | Jan. | 1 | 5 | 0 |
| 9 | P. Eye, 21st | May | 1 | 12 | 0 |

* P. = primary tumor.

† M. = lung metastasis.

A total of 32 males were used and 6.2 per cent of takes resulted, whereas no growth occurred in an equal number of females. The takes were obtained in June, 1938, and in June, 1939, with material derived from the 2nd eye generation of the primary growth and the 8th eye generation of the metastasis. Serial transfers were attempted in both instances but growth failed to occur in the 2nd generation.

Growth of the transplants was evident in both cases on the 25th day. The fragments increased slowly in size and were distinguished by the same cherry red color that characterizes growths of this tumor in the rabbit. One animal was killed on the 42nd day when the chamber was one-fourth filled with tumor, while the other was held until the growth filled one-half the chamber on the 100th day.

Microscopically, the guinea pig tumors were identical with those resulting from transfer to the rabbit's eye and consisted of an intricate arrangement of interlacing strands of epithelium and connective tissue carrying a rich supply of blood vessels (Figs.

11 and 12). In both instances, the tumors had invaded the iris and ciliary body, but neither had extended to the posterior chamber. No metastases were found.

Brown-Pearce Tumor

Six attempts were made to transfer the Brown-Pearce rabbit tumor to guinea pigs, but all terminated in failure. The tumor used was obtained from a testicular transplant and first transferred to the eyes of rabbits. Takes occurred in 100 per cent of animals and the tumor was carried for six serial generations in this site. Attempts were made to transfer the growth from representative animals of each generation to the anterior chambers of the eyes of guinea pigs. The uniformly unsuccessful results are recorded in Table IV.

TABLE IV

The Results of Transplantation of the Brown-Pearce Rabbit Carcinoma into the Anterior Chamber of the Eyes of Guinea Pigs

| Series No. | Source and generation of rabbit tumor | Date of transfer | Guinea pigs | | Total takes |
|------------|---------------------------------------|------------------|----------------|-------------|-------------|
| | | | Generation No. | Number used | |
| | | 1939 | | | per cent |
| 1 | Eye, 1st | Nov. | 1 | 6 | 0 |
| 2 | " 2nd | " | 1 | 11 | 0 |
| 3 | " 3rd | Dec. | 1 | 4 | 0 |
| | | 1940 | | | |
| 4 | " 4th | Jan. | 1 | 5 | 0 |
| 5 | " 5th | " | 1 | 4 | 0 |
| 6 | " 6th | Feb. | 1 | 5 | 0 |

Normal Rabbit Tissues

The question arose following the successful heterologous transplantation of rabbit tumors as to whether the ability to survive and grow in a foreign species is a peculiar property of neoplastic tissue or is also shared by normal tissue. The ability of normal tissues to grow in the anterior chambers of animals of the same species is well recognized and forms the basis of numerous experimental investigations. Prior to the present work it was observed in this laboratory that endometrium or breast tissue obtained from normal adult rabbits as well as embryonic parts containing mixed tissues could be grown indefinitely in the anterior chambers of the eyes of other rabbits. Adult tissue was found to increase in size at a very slow rate, and no attempt was made to carry the tissue for more than a single generation. On the other hand, embryonic tissue grows rapidly, and in one experiment fragments derived from a 10 day old fetus were carried for

six serial generations. Experiments were performed to determine whether or not these tissues could be grown in the anterior chambers of guinea pigs' eyes.

Fragments of normal adult rabbit endometrium were transferred to the eyes of nine guinea pigs. On the 7th day the transplants in eight of the animals were pinkish in color and apparently living, while in one animal the fragment was opaque, white, and obviously dead. The fragments were still living on the 19th day, and histological examination showed the presence of a vascular supply and the absence of any degenerative process or foreign body reaction. However, by the 25th day the pinkish coloration had disappeared and portions of the transplants were opaque and white. The animals were killed on the 28th day, and on microscopic examination the fragments were found to be in an advanced state of degeneration.

Almost identical results were obtained on transplantation of normal adult breast tissue. Embryonic tissue, on the other hand, survived and increased in size until the animals were killed at the end of a month. The rate of growth in guinea pigs, however, was very much slower than in the rabbit, and the fragments had only doubled in diameter when the experiment was terminated. Moreover, histological examination showed that survival and growth were limited to skin and cartilage and that all other tissues of the original graft had disappeared.

SUMMARY

A series of experiments was undertaken in an attempt to transplant rabbit tumors to animals of alien species using the anterior chamber of the eye as an inoculation site. The uterine tumor H-31 and the breast tumors T-36 and B-240 were successfully transplanted to all the various animals tried, including guinea pigs, swine, goats, and sheep, and the H-31 and T-36 tumors were maintained by serial transfer in the two first species. On the other hand, all attempts to transfer the Brown-Pearce tumor to guinea pigs were unsuccessful.

The growth characteristics of the transplanted tumors were generally similar to those observed in the natural host, but noteworthy exceptions occurred. The tumors obtained a blood supply from the foreign host and invaded the periorbital tissues but did not metastasize. Histologically, the cellular morphology of the rabbit tumors was retained, but variations in parenchymal-stromal relations characterized the form of growth in different species.

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EXPLANATION OF PLATES

PLATE 22

FIG. 1. Section of a transplant of the rabbit uterine cancer H-31 from the eye of a first generation guinea pig. The animal was killed 48 days after transfer, and at autopsy, the anterior chamber was one-half filled with tumor. The acinar arrangement of cells is identical with that frequently observed in growths of the same tumor in the rabbit's eye. Hematoxylin and eosin. $\times 225$.

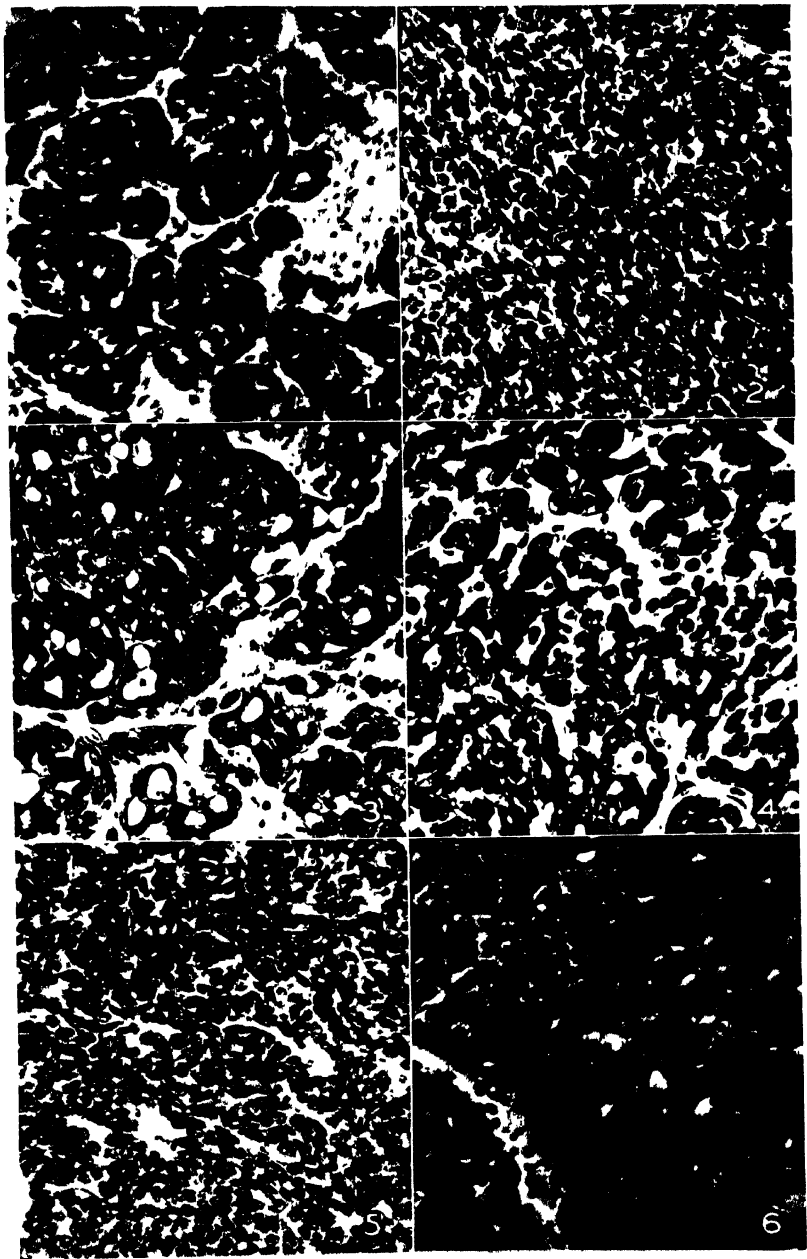
FIG. 2. Section of a transplant of the H-31 rabbit tumor from the eye of a third generation guinea pig. The animal was killed 78 days after transfer, and at autopsy, the anterior chamber was completely filled with tumor. Solid cellular growths without structural organization also occur in the rabbit. Hematoxylin and eosin. $\times 225$.

FIG. 3. Section of a transplant of the H-31 rabbit tumor from the eye of a fourth generation guinea pig. The animal was killed on the 390th day after transfer and the chamber was two-thirds filled with tumor. Sharply outlined cells with condensation of cytoplasm giving rise to "signet ring" acini were a characteristic feature of many older growths. Hematoxylin and eosin. $\times 225$.

FIG. 4. Section of a transplant of the H-31 rabbit tumor from the eye of a fourth generation guinea pig. The animal was killed 509 days after transfer and during this period the transplant had increased by only half a diameter. Cells appeared as isolated units in a dense hyaline stroma. Hematoxylin and eosin. $\times 225$.

FIG. 5. Section of the tumor arising in the eye of a rabbit from transplantation of a fragment of the guinea pig growth shown in Fig. 4. The rabbit was killed 66 days after transfer and the chamber was completely filled. The type of growth is identical with that observed in serial rabbit transfers. Hematoxylin and eosin. $\times 225$.

FIG. 6. Section of a transplant of the H-31 rabbit tumor from the eye of a first generation hog. The chamber was completely filled on the 15th day after transfer and the tumor was removed at biopsy. Growth occurred in solid cellular sheets and there was no sign of acinar formation. Hematoxylin and eosin. $\times 225$.



Photographed by J A Carlile

(Greene Heterologous transplantation of mammalian tumors 1)

PLATE 23

FIG. 7. Section of a transplant of the rabbit breast cancer T-36 from the eye of a 12th generation rabbit. The animal was killed on the 13th day and the chamber was one-third filled with tumor. Growth in solid cellular sheets without structural pattern was a constant feature of the tumor in this species. Hematoxylin and eosin. $\times 225$.

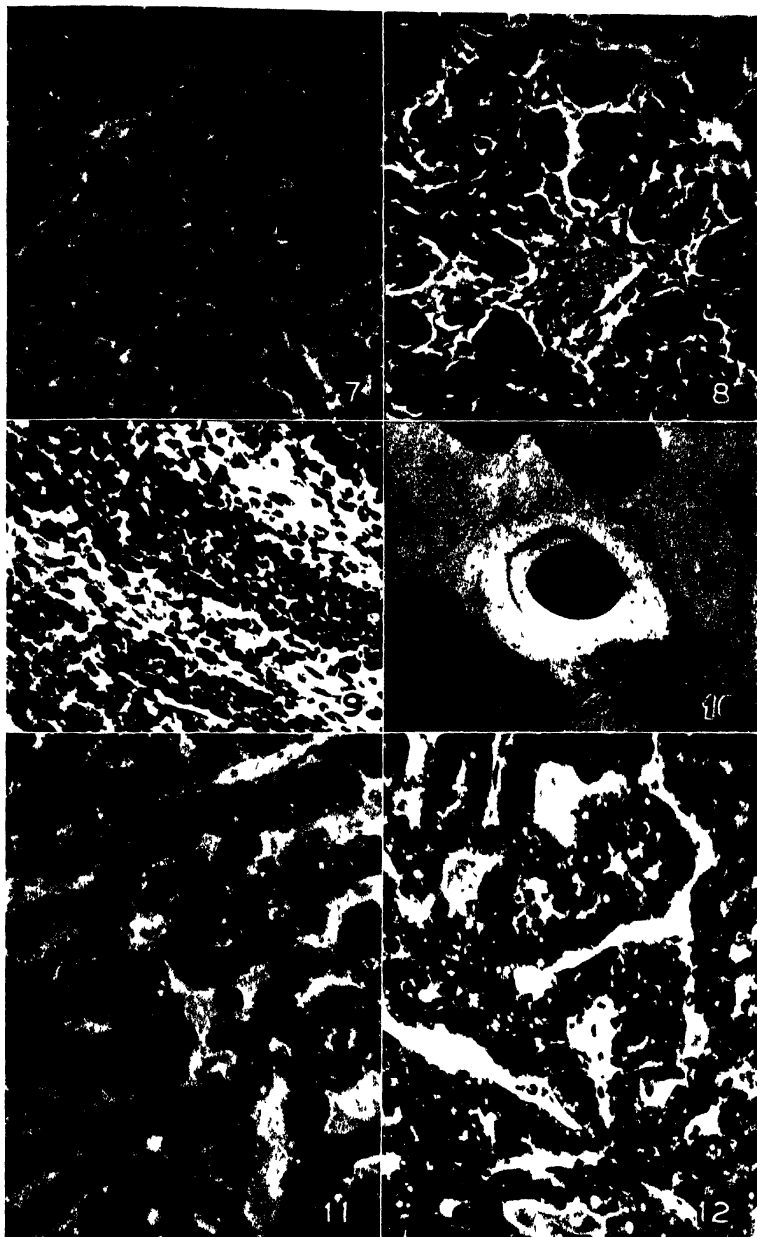
FIG. 8. Section of a transplant of the T-36 rabbit tumor from the eye of a tenth generation guinea pig. The animal was killed on the 10th day and the chamber was three-quarters filled. The formation of small acinar bundles was a characteristic of growth in the guinea pig. A number of thin-walled blood vessels can be seen in the photograph. Hematoxylin and eosin. $\times 225$.

FIG. 9. Section of a transplant of the T-36 rabbit tumor from the eye of a seventh generation guinea pig killed 84 days after transfer. The growth had invaded the cornea and extended to periorbital tissues. Section shows infiltrating columns of tumor cells. Hematoxylin and eosin. $\times 225$.

FIG. 10. Eye of a first generation hog bearing a transplant of the T-36 rabbit tumor photographed 11 days after transfer. The fragment measured 1 mm. in diameter at transfer and increased to fill the chamber in 23 days. Actual size.

FIG. 11. Section of a transplant of the rabbit breast cancer B-240 from the eye of a fifth generation rabbit. The animal was killed 69 days after transfer when the chamber was one-quarter filled. Hematoxylin and eosin. $\times 225$.

FIG. 12. Section of a transplant of the B-240 rabbit tumor from the eye of a first generation guinea pig killed on the 100th day after transfer when the chamber was one-half filled with tumor. The structure is identical with that found in the rabbit. Hematoxylin and eosin. $\times 225$.



Photographed by J A Carlile

(Greene Heterologous transplantation of mammalian tumors I)

HETEROLOGOUS TRANSPLANTATION OF MAMMALIAN TUMORS

II. THE TRANSFER OF HUMAN TUMORS TO ALIEN SPECIES

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PLATES 24 AND 25

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The successful transplantation of rabbit tumors into the anterior chamber of the eyes of guinea pigs and other alien species suggested an attempt to transplant human tumors to lower animals using the same method of transfer. In the experiments to be reported, transplantation of a number of human cancers to guinea pigs and rabbits was effected and in one instance the transplanted tumor has been maintained by serial transfer. The object of the present paper is to record the results of this series of experiments and to describe the growth characteristics of the tumors in the alien species.

Materials and Methods

The present experiments are concerned with attempts to transplant eleven different human tumors, including eight carcinomata and three sarcomata (Tables I and II).

The tumors consisted entirely of surgical material and were obtained from hospitals in New York, Philadelphia, New Haven, and Trenton.¹ Tumor fragments were transported from the operating room in test tubes in a cooled thermos bottle and transplanted immediately upon arrival at the laboratory. As a rule, transplantation was completed within 2 to 4 hours of operation, but in one instance in which successful transfer was effected, material sent by railway express from New Haven remained in transit for 30 hours.

In contrast to the procedure utilized in the heterologous transplantation of rabbit

¹ The author wishes to acknowledge the cooperation of Dr. S. P. Reimann of the Lankenau Hospital, Philadelphia; Dr. M. C. Winternitz and Dr. Robert Tennant of the Yale University School of Medicine; Dr. R. A. Moore of Cornell University Medical College; Dr. R. L. Pfeiffer of the Eye Institute, Presbyterian Hospital, Columbia University; and Dr. E. L. Shaffer of the St. Francis Hospital, Trenton, New Jersey, in making fresh human tumor material available for this study.

tumors which included serial passage in the rabbit's eye before transfer to the foreign species, human material was of necessity transferred directly from the primary host. Rabbits and guinea pigs were used exclusively as experimental animals. The rabbits were hybrids ranging from 6 months to 2 years in age, while the guinea pigs varied from 250 to 1000 gm. in weight. The animals were not subjected to special treatment either before or after transfer but were maintained under ordinary conditions of cage life.

The technique utilized in the transplantation of the tumors was identical with that used in the transfer of rabbit tumors and its essential features were reviewed in the preceding paper.²

Tissues for microscopic examination were fixed in Petrunkevitch's solution and stained with hematoxylin and eosin.

Carcinomata

The epithelial tumors used consisted of cancers of the breast, uterine fundus, cervix, colon, and tongue. Four mammary cancers were transplanted and all were of the scirrhus variety. The cancer of the fundus was an adenocarcinoma, the cervical growth a transitional cell epithelioma, the tumor of the colon a stenosing fibrocarcinoma, and the tongue cancer an epidermoid carcinoma. With the exception of the uterine adenocarcinoma which was made up almost entirely of glandular elements, the tumor fragments received in the laboratory consisted largely of dense, fibrous, connective tissue, and in many cases it was doubtful at transfer whether or not essential tumor elements were included in the transplants.

The results of transplantation are shown in Table I. The cancers of the tongue, colon, cervix, and fundus were infected and with a single exception it was necessary to discard all animals bearing transplants within a week of transfer. The exception consisted of a guinea pig of the colon series, and in this instance the eye was not infected and the fragment grew. Growth also occurred in fragments derived from two different mammary tumors.

Mammary Cancers.—Fragments derived from two of the mammary cancers failed to grow but takes resulted from transfer to rabbits of the two tumors obtained in June and September, 1938. A total of 37.5 per cent of the latter transplants increased in size and showed other presumptive signs of life, but with two exceptions growth ceased and regression occurred at the end of 3 months. The two exceptions were in animals implanted in June and in both instances the transplants were actively growing when the animals were killed 5 months later.

² Greene, H. S. N., *J. Exp. Med.*, 1941, **73**, 461.

The early manifestations of growth were similar in all cases. The fragments remained opaque white in color and completely inactive during the first 3 weeks after transfer. At the end of this period, however, a pinkish coloration and a slight increase in size distinguished living transplants. Growth continued in such cases but was always limited to one pole or to the edges of the fragment and a portion of the graft remained opaque white in appearance.

The fragments doubled in diameter during the first 2 months and toward the end of this period vascularization became evident. The vascular supply was derived from the iris and its extension through the graft was accompanied by an ingrowth of pigmented cells.

Regressive changes appeared in seven of the transplants when they had attained a diameter of approximately 5 mm. and a gradual decrease in size became evident. Even-

TABLE I
The Results of the Heterologous Transplantation of Human Carcinomata

| Material | Date of transfer | Interval between operation and transfer | Rabbits | | | Guinea pigs | | |
|---------------------------------------|------------------|-----------------------------------------|-------------|----------------------------------------------|----------------------------------|-------------|----------------------------------------------|----------------------------------|
| | | | Number used | Number showing growth followed by regression | Number showing persistent growth | Number used | Number showing growth followed by regression | Number showing persistent growth |
| | | | | | | | | |
| Scirrhus carcinoma of breast | 1938 | hrs | | | | | | |
| | June | 2 | 12 | 5 | 2 | | | |
| | Sept. | 3 | 12 | 2 | 0 | | | |
| " " " " | Oct. | 5 | 7 | 0 | 0 | | | |
| | 1939 | | | | | | | |
| " " " " | Sept. | 2 | 12 | 0 | 0 | 11 | 0 | 0 |
| | Nov. | 2 | | | | 8 | 0 | 1 |
| Stenosing carcinoma of colon | | | | | | | | |
| Adenocarcinoma of uterine fundus | Apr. | 2 | 8 | Infected | | | | |
| Transitional cell carcinoma of cervix | Sept. | 2 | 10 | " | | 6 | Infected | |
| Epidermoid carcinoma of tongue | Oct. | 2 | 10 | " | | | | |

tually the tumors contracted to yellowish-brown, smooth masses of hyaline substance less than one-half their former diameter and remained unchanged in that condition.

On the contrary, growth persisted in two of the animals (Fig. 1). In one, the rate was slower from the very beginning and the tumor had only increased to 3 mm. in diameter at the end of the 3rd month. However, regression did not occur and when the animal was killed 5 months after transfer, the growth had formed an oval mass 6 mm. in diameter occupying nearly half of the chamber. Fragments of this tumor were transferred to the eyes of a second generation but no takes resulted.

In the second instance, the growth was more invasive than expansive in character and infiltrated the iris over a wider area. At the end of the 3rd month, a crater-like depression developed in the center of the growth and gradually deepened. Subsequently, a perforation appeared through the remains of the underlying iris and extended to form a wide opening into the posterior chamber of the same general size and appearance as the contracted pupil. Simultaneously, the cornea was invaded and for a period of

time extension to the outside seemed imminent. However, perforation did not occur and a shell of cornea approximately one-fifth the normal thickness of the tissue covered the growth at autopsy. The tumor showed little further increase in size but remained living until the animal was killed at the end of 5 months.

At autopsy, no evidence of regression was found in either case. In the first instance, the tumor was attached to the iris by a short, thin pedicle containing many blood vessels and, despite intimate contact in other regions, invasion was limited to this small area. The growth was soft and medullary in consistency and section revealed a smooth, homogeneous surface. In the second instance, tumor tissue was limited to the edges of the ulcer where it was piled up and densely adherent to the cornea.

Histologically, the cells of the transplants contained less cytoplasm and their nuclei were rounder and less vesicular than those of the primary tumor. Moreover, the pronounced connective tissue reaction that characterized the growth in the spontaneous human host was completely lacking and epithelial cells were arranged in a solid medullary mass (Figs. 2-4). Blood vessels were plentiful and there were no degenerative changes. No gross or microscopic metastases were found.

Cancer of the Colon.—At operation, samples of the tumor were secured from the periphery of the growth at some distance from the lumen of the colon, but despite this precaution, all but one of the fragments proved to be infected. In this single instance, no sign of infection developed and indications of growth became evident after 2 weeks residence in the anterior chamber of a guinea pig.

Growth was more rapid than was observed in the case of the previous transplants, and at the end of 2 months, the chamber was one-half filled. The tumor was gray rather than pink in color and its surface was irregular, with finger-like projections of tissue, in contrast to the smooth appearance of other transplants. Approximately 3 months after transfer, when the mass measured 5 mm. in diameter, an opaque white zone appeared at one pole and, because of this suggestion of beginning regression, the animal was killed for histological study.

At autopsy, the growth was intimately attached to the iris along its entire posterior surface and could not be separated from this structure without tearing. Microscopically, its cells were larger with more cytoplasm and less nuclear substance than those of the original tumor. There was no attempt at tubule formation and cells were arranged in unorganized sheets (Figs. 5 and 6). The intense connective tissue reaction of the spontaneous growth was not duplicated but the transplant contained more connective tissue than was noted in the mammary cancer transplants and in several regions thick fibrous bands encircled islands of cells. The transplant was well supplied with blood vessels but there were scattered areas of degeneration and necrosis. No metastases were found.

Sarcomata

Attempts were made to transfer three different human sarcomata consisting of a retinoblastoma, a melanotic sarcoma, and a fibrosarcoma. The retinoblastoma was a glial sarcoma of extremely soft, almost gelatinous consistency, containing many areas of fatty degeneration and numerous foci

of calcification. The second tumor was a metastasis of a primary melanotic sarcoma of the skin of the face and was obtained from the chest wall at operation. Neither gross nor microscopic pigment was present in the fragments used for transfer. The fibrosarcoma originated in the breast of a 20 year old woman. The greater part of the growth had been removed during an exploratory operation, and following identification of the tumor, a total mastectomy was performed. The tissue used for transfer was obtained at the mastectomy and consisted of a nodule approximately 4 mm. in diameter.

The results of transplantation are shown in Table II. No takes followed transfer of the retinoblastoma but, as noted above, the tumor was in an advanced stage of degeneration. On the contrary, takes resulted

TABLE II
The Results of the Heterologous Transplantation of Human Sarcomata

| Material | Heterologous generation number | Date of transfer | Interval between operation and transfer | Rabbits | | Guinea pigs | |
|-------------------------------------------|--------------------------------|------------------|-----------------------------------------|-------------|----------------------------------|-------------|----------------------------------|
| | | | | Number used | Number showing persistent growth | Number used | Number showing persistent growth |
| | | 1939 | hrs. | | | | |
| Retinoblastoma | 1 | Jan. | 4 | 11 | 0 | | |
| Metastasis from melanotic sarcoma of skin | 1 | Feb. | 30 | 5 | 0 | 4 | 1 |
| | 2 | Aug. | | | | 8 | 0 |
| Fibrosarcoma of breast | 1 | Mar. | 2 | | | 20 | 4 |

from transplantation of the melanotic tumor and the fibrosarcoma, and in no instance did regression of the resulting growth occur. Moreover, transfer to a second generation was successfully effected in the case of the fibrosarcoma.

Retinoblastoma.—The transplanted fragments of this tumor rapidly disappeared and no indication of their presence could be found in the eyes of the experimental rabbits on the 5th day after transfer. The animals were held under observation for $2\frac{1}{2}$ months and throughout this period the same condition prevailed.

Melanotic Sarcoma.—The transplants of this tumor remained unchanged during the first 3 months following transfer, and at the end of the period all of the rabbits bearing transplants were discarded. The guinea pigs were held for further observation and at $3\frac{1}{2}$ months alterations suggestive of growth were observed in the eye of one animal.

The entire substance of the tumor fragment became pink in color and examination with a magnifying lens showed the presence of surface vessels. A rapid increase in size was then observed and at the end of 2 weeks the transplant had tripled in diameter. A period of quiescence followed with no other change in appearance, but after a month growth was renewed and persisted at a uniform rate until the animal was killed with the chamber one-half filled at the end of 6 months (Fig. 7).

The growing tumor was characterized throughout its course by the presence of surface blood vessels visible in the gross and by a persistent semitranslucent pinkish appearance. At an early period of growth, a temporary brownish tinge suggested the production of melanin, but the coloration later disappeared and its occurrence may have been related to the resorption of hemorrhage.

The point is of particular interest inasmuch as both the original tumor tissue and the iris of the experimental animal were unpigmented and the occurrence of melanin in the transplanted growth would have been evidence of the synthesis of that pigment. The probability that the brown discoloration arose from the disintegration of red blood cells is enhanced by the fact that shortly before the animal was killed a large, visible hemorrhage did occur. The hemorrhage filled the remaining half of the chamber and was the immediate reason for the termination of the experiment.

The hemorrhage undoubtedly resulted from the invasion of vascular walls with subsequent rupture, for the invasability of the growth was attested at autopsy by the complete destruction of the underlying iris and ciliary body. The tumor, however, was limited to the eyeball and had not invaded the sclera. Microscopically, the cells and structure of the growth were identical with those of the spontaneous human tumor (Figs. 8 and 9). Neither gross nor microscopic metastases were found.

Fragments of the tumor were transferred to the eyes of eight additional guinea pigs. The animals were held under observation for 7 months but no growth occurred.

Fibrosarcoma.—As noted above, the amount of this tumor available for transfer was extremely small and only a 4 mm. nodule in the breast tissue obtained at operation showed the gross characteristics of sarcoma. A portion of the nodule was used for frozen section, while the remainder was divided into fragments and transferred to the anterior chambers of four guinea pigs. In addition, fragments of adjacent normal appearing breast tissue were used for transfer in the hope that they contained invading sarcoma cells. Takes occurred in all of the four pigs that received sarcoma tissue, but to date, no evidence of growth has been observed in the other animals. Thus, while examination of Table II suggests that there were only 20 per cent of takes, in actuality there occurred 100 per cent of takes in cases in which known sarcoma tissue was used.

Growth was evident in two of the animals on the 44th day but in the remaining two the fragments remained unchanged for 130 days. The former pigs were both killed on the 123rd day when the growth filled one-third of the chamber. One of the latter pigs was killed on the 162nd day with a growth of the same size, while the remaining animal is still living (200 days) and bears a growth which fills one-half the chamber (Figs. 10 and 11).

The characteristics of the tumors in the anterior chamber were similar to those of the melanotic sarcoma but, in general, growth proceeded at a more uniform and rapid rate. The transplants increased in all diameters and invasion of the iris with extension into the posterior chamber was an early occurrence. Thus, in contrast to rabbit tumors which grew laterally and filled the chamber before extending posteriorly, the portion of sarcoma transplants in the posterior chamber at any given time was approximately equal in size to that present in the anterior chamber.

The tumors were supplied with large blood vessels which could be seen traversing the surface with the naked eye. The tissue remained healthy in all cases and there were no changes suggestive of degeneration or regression throughout the life of the animal.

At autopsy, the tumors protruded through the iris into the posterior chamber but remained discrete and did not invade other structures. They were oval in shape, firm in consistency, and appeared homogeneous on section. Histologically, their cells and structure were identical with those of the primary human tumor (Figs. 12 and 13). No metastases were found.

Serial transplantation was attempted with tumor material obtained from one of the animals killed on the 123rd day. Ten guinea pigs were used, and while it is still too early to gauge the final outcome of the experiment (66 days after transfer), definite growth has occurred in two animals, two others show presumptive evidence of growth, and the fragments are unchanged in the remainder.

Serial transfer was also performed with material from an animal killed on the 162nd day. Twenty-four guinea pigs were used and at the present time, 27 days after transfer, there is definite growth in three, presumptive evidence of growth in sixteen, and no evidence of change in the remainder.

DISCUSSION

In the present series of experiments, rabbit tumors were transplanted to animals of all the various species used, including guinea pigs, swine, sheep, and goats, and in addition, several human tumors were successfully transferred to rabbits and guinea pigs. Serial transplantation in the alien host was successfully carried out in both cases. The anterior chamber of the eye was used as an implantation site, and in all instances transfer to other regions was unsuccessful. The success which attended the experiments in contrast to the failure that followed attempts to use other sites requires some comment.

In all tumor transfers it is clear that the transplanted cells must live for a period of time in the manner of a tissue culture; tumor cells may subsist for a time on materials introduced with the inoculum, but continued survival is dependent upon the imbibition of nutrient from the surrounding tissue fluids. A foreign body reaction is not immediate but occurs after a variable interval and in variable intensity depending on the constitution

of the transplant and the host and on the sensitivity of the region used as an implantation site. In ordinary homologous transfers involving tissues of close generic relationship, the foreign body reaction is inconspicuous and consists largely in a proliferation of fixed tissue elements which eventually results in vascularization of the fragment. On the contrary, the transfer of foreign tissue invokes an intense reaction. The fragment acts as an inflammatory stimulus, free mobile elements of the blood and lymph migrate to the tissue in great numbers and the fixed tissue proliferation results in encapsulation rather than in vascularization.

However, if for some reason the foreign body reaction is delayed and the transplant lives, it is conceivable that its cells may be gradually altered by the continued imbibition of host materials so that some degree of adaptation results and the more radical constitutional differences between transplant and host are lost. The eventual foreign body reaction would then be less intense and more in the nature of that which follows homologous transplantation.

It is possible that such a sequence occurs in the anterior chamber of the eye. The iris is apparently relatively unresponsive to the presence of foreign bodies and an interaction between the tissues of the transplant and the host is delayed for a considerable period of time. During the interim, the transplanted fragment persists as a free entity deriving nourishment from the fluid of the chamber and its cells remain entirely independent of the tissues of the host. Growth proceeds during this culture-like phase and an increase in size is evident before the occurrence of vascularization. The inflammatory reaction which follows the introduction of foreign tissue into other regions of the body does not occur. On the contrary the transplant,—which at first survives as a parasitic tissue culture,—becomes supplied after a time with a rich plexus of blood vessels, is incorporated in the body of the host, and enters into intimate relations with the organism, as if transplanted to an animal of the same species. This point deserves emphasis.

The general behavior of the rabbit tumors in the foreign host is comparable with that observed in experimental animals of the same species. Invasions of normal tissues occur but usually expansive growth proceeds at a greater rate and the increased pressure incident to the rapid filling of the chamber interferes with the blood supply and regression follows. Metastasis has not been observed but it should be noted that the rabbit tumor most extensively employed in heterologous transfers (T-36) has never given rise to metastatic growths in the rabbit. On the other hand, variations from the behavior observed in rabbits do occur and are of particular in-

terest. The H-31 tumor occasionally grows at an extremely slow rate and persists for longer periods of time in guinea pigs than has ever been noted in homologous transplantation experiments, but the same tumor grows much more rapidly in hogs than in the natural host. Likewise, the T-36 tumor proliferates much more rapidly in guinea pigs and occasionally grows progressively in this species, in sharp contrast to its behavior in the rabbit.

Morphologically, the structure of the essential epithelial elements of the rabbit tumors remains unchanged in heterologous species but variations in the parenchymal-stromal relations suggesting a higher degree of organization characterize the appearance of the T-36 tumor in the guinea pig. Such a finding constitutes an exception to the general findings on tumor form and behavior according to which organization and growth rate are inversely related. Here, however, a higher organization is associated with an increased growth rate.

No criteria exist for a comparison of the behavior of human tumors in the eyes of alien species. However, the long period of quiescence which follows transfer to the new species is suggestive of the interval which frequently separates surgical removal and local recurrence in the human host. The fluctuation in growth rate which characterized several of the tumors is also suggestive of their behavior under natural conditions.

Morphologically, the transplants of the two carcinomata differ so radically in appearance from the spontaneous tumors that some doubt of their identity is justified. However, in both instances the spontaneous tumors owed their characteristic appearance to desmoplastic properties and the abundant fibrous tissue which gave them a special structure was not essential stroma but rather a local reaction to their presence. The absence of large amounts of connective tissue in the transplants may thus be interpreted as a failure of the tumor to elicit the same response in the animal's eye as was provoked in the region of spontaneous growth. And, in actuality, there is no reason to expect reactions in the eye of the rabbit to duplicate those that occur in the colon or breast of man. The best criteria of the manner in which such tumors might grow in the anterior chamber of man are perhaps to be found in the spontaneous growths of the iris and here pronounced cellularity with little fixed tissue reaction is the rule. No precedent exists in morphology upon which to base proof of the successful heterologous transplantation of human carcinomata; and in the present instances, the burden of evidence consists rather in the directly observed growth of transplanted fragments and the presence of invasion in microscopic sections.

Questions of this nature do not apply to the transplantation of the two human sarcomata for in both instances the transplants and the spontaneous tumors were morphologically identical in appearance. Moreover, the fibrosarcoma was successfully transferred to a second generation.

The long latent period which sometimes followed transfer of the human sarcomata or of the rabbit carcinoma H-31 warrants further consideration. During this period of inactivity which lasted for as long as 4 months the transplants received no blood supply and stimulated no reaction in the iris, yet eventually grew and expressed their cancerous nature. Occasionally, in transplants of the H-31 tumor, the fragments remained relatively inert for more than 500 days but when removed from the guinea pig and replaced in the rabbit, there occurred an almost immediate reversion to typical growth characteristics. Presumably, cell division ceased entirely or was tremendously retarded in response to some influence associated with transfer to the new environment and this constitutes further evidence that the growth of cancer can be controlled without destruction of its essential cells. A similar phenomenon is frequently observed in man when, after removal of a tumor, displaced cells remain quiescent for long periods of time, often for years, but finally express their potentialities and lead to death. The factors concerned are open to experimental study and would appear to be of considerable importance in the control of malignancy.

The failure to transfer the Brown-Pearce tumor to guinea pigs likewise requires some comment. An adequate test was performed and the conclusion that the growth is not transplantable to this species by the methods employed appears to be justified. Heterologous transplantation of rabbit tumors has failed in only one other instance and the two tumors have a factor in common. The H-31 tumor was easily transferred from early rabbit generations, but despite numerous attempts, transfers from later generations were all unsuccessful. The Brown-Pearce tumor has been carried by serial transfer in the rabbit since 1921. Thus, a feature shared by the two non-transplantable growths consists of continued serial passage in the same species over a relatively long period of time, and it is conceivable that this common attribute may be associated with their non-transplantability to a foreign species.

In any case, it has been demonstrated that in certain instances human as well as rabbit tumors can be transplanted to alien species and maintained by serial passage in the foreign host. This point of itself is of considerable importance to biology and cancer research and opens up a number of interesting problems. The ability to grow indefinitely in the eye of an alien host is apparently a property peculiar to cancerous tissue. Normal

adult tissue possesses a restricted capacity in this direction, while the capacity of embryonic material is somewhat greater but still sharply limited.

SUMMARY

The successful heterologous transplantation of several human tumors, including a breast carcinoma, a carcinoma of the colon, a melanotic sarcoma, and a fibrosarcoma, has been reported. Histologically, the appearance of the transplants of the carcinomata differed from that of the spontaneous tumors, but the directly observed growth of the transplanted fragments together with the presence of invasion in microscopic sections afforded proof of successful transfer. Transplants of the sarcomata, on the other hand, were morphologically identical with the primary tumors, and in the case of the fibrosarcoma serial passage to a second generation of animals was successfully performed.

EXPLANATION OF PLATES

PLATE 24

FIG. 1. Eye of a rabbit containing a growing transplant of a human mammary carcinoma. The photograph was taken $4\frac{1}{2}$ months after transfer of a fragment of tumor tissue measuring 1 mm. in diameter and during the interim the transplant had undergone a sixfold increase in size. Actual size.

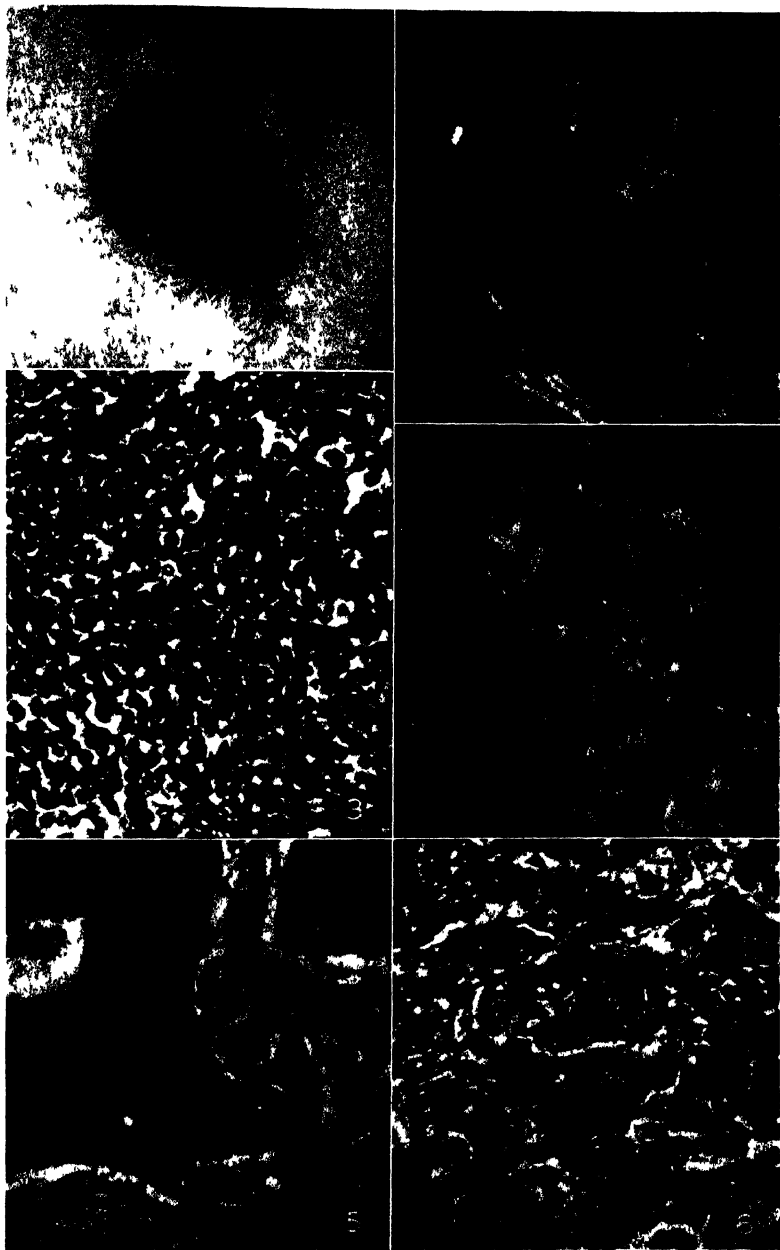
FIG. 2. Section of the original scirrhous mammary carcinoma transplanted to the rabbit pictured in Fig. 1. Hematoxylin and eosin. $\times 406.5$.

FIG. 3. Section of the transplant from the eye of the rabbit shown in Fig. 1. The animal was killed 5 months after transfer. Morphologically, the connective tissue reaction which characterized the spontaneous tumor is absent in the rabbit, but the essential epithelial elements of the two growths are almost identical. Hematoxylin and eosin. $\times 406.5$.

FIG. 4. Section of a transplanted fragment of the same mammary cancer that remained unchanged in the eye of another rabbit for 5 months. Compare with Fig. 3. Hematoxylin and eosin. $\times 406.5$.

FIG. 5. Section of the cancer of the colon from which the transplant in Fig. 6 was derived. Hematoxylin and eosin. $\times 406.5$.

FIG. 6. Section of a tumor in a guinea pig's eye resulting from transfer of the human cancer shown in Fig. 5. Histologically, there is no attempt at organization and the cells are generally larger than those of the human tumor. Hematoxylin and eosin. $\times 406.5$.



Photographed by J. A. Carlile

(Greene: Heterologous transplantation of mammalian tumors 11)

PLATE 25

FIG. 7. Eye of a guinea pig containing a growing transplant of a human melanotic sarcoma. The lateral half of the anterior chamber is almost completely filled with tumor. Photograph was taken 5½ months after transfer of a fragment measuring 1 mm. in diameter. Actual size.

FIG. 8. Section of the human melanotic sarcoma transplanted to the guinea pig shown in Fig. 7. Hematoxylin and eosin. $\times 282$.

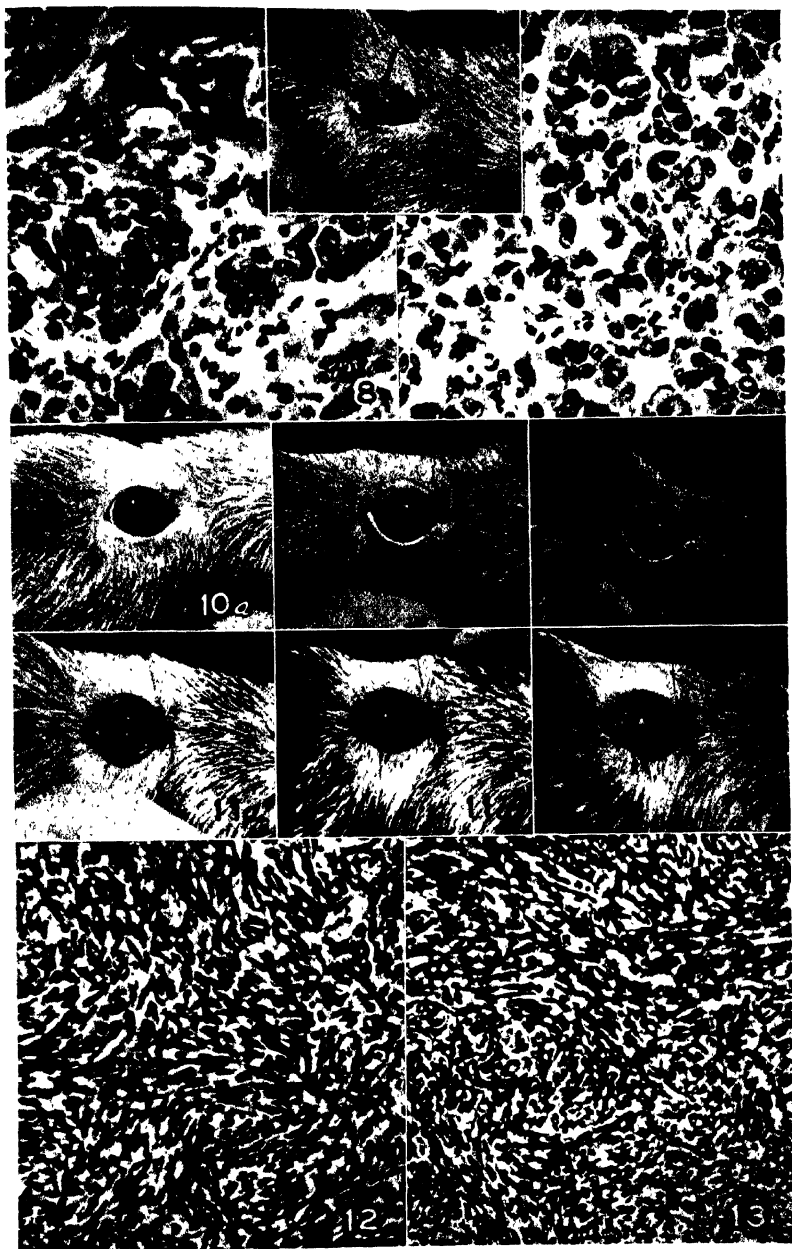
FIG. 9. Section of the transplant from the eye of the guinea pig shown in Fig. 7. Histologically, the guinea pig transplant and the human tumor are identical in appearance. Hematoxylin and eosin. $\times 282$.

FIG. 10. Eye of a guinea pig containing a growing transplant of a human fibrosarcoma. In this instance, growth was evident on the 44th day after transfer. (a) Appearance of the transplant on the 82nd day after transfer, (b) the 98th day, and (c) the 113th day. Actual size.

FIG. 11. Eye of a guinea pig containing a growing transplant of the same human fibrosarcoma. Growth in this case was not apparent until the 130th day after transfer. (a) Appearance of the transplant on the 166th day after transfer, (b) the 180th day, and (c) the 187th day. This animal is still living (200 days after transfer), and the transplant fills one-half of the chamber. Actual size.

FIG. 12. Section of the human fibrosarcoma transplanted to the guinea pigs shown in Figs. 10 and 11. Frozen section. Hematoxylin and eosin. $\times 225$.

FIG. 13. Section of the transplant of the human fibrosarcoma in the eye of the guinea pig shown in Fig. 10. The human tumor and guinea pig transplant are morphologically identical. Hematoxylin and eosin. $\times 225$.



Photographed by J. A. Carlile

(Greene Heterologous transplantation of mammalian tumors II)

THE SWINE LUNGWORM AS A RESERVOIR AND INTERMEDIATE HOST FOR SWINE INFLUENZA VIRUS

I. THE PRESENCE OF SWINE INFLUENZA VIRUS IN HEALTHY AND SUSCEPTIBLE PIGS

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In this series of papers data will be presented which demonstrate the fact that the swine lungworm serves under natural conditions as a reservoir and intermediate host for the swine influenza virus. The virus can persist in a masked form within its worm host for long periods of time, and months or even years may elapse between its transmission from one swine to the next. The period during which the virus survives in the lungworm is more than adequate to account for its persistence between epizootics of swine influenza. In this first paper facts will be presented which show that healthy susceptible pigs sometimes contain the swine influenza virus and may undergo attacks of influenza if the virus is provoked to activity by multiple intramuscular injections of the bacterium *Hemophilus influenzae suis* (1).

Of the two agents which act in concert to cause swine influenza (1), it has been shown that the bacterial component, *H. influenzae suis*, is capable of eliciting an immune response that affords only partial protection against the disease (2). Swine influenza virus vaccines, on the other hand, confer a complete immunity to swine influenza (3). The observations now to be recorded were made as a result of further study of the use of *H. influenzae suis* vaccines in the prophylaxis of swine influenza.

Swine Influenza Precipitated by Inoculation with Hemophilus influenzae suis Vaccines

Preparation of H. influenzae suis Vaccines.—Cultures 18, 23, and 28 *H. influenzae suis*, originally obtained from naturally occurring field cases of swine influenza, were pooled for use in the experiments. The 48 hour growths from potato extract-chocolate agar slants were scraped off and suspended in a small amount of physiological saline. These suspensions were then centrifuged in graduated tubes for $\frac{1}{2}$ hour at 1600 to 1800 R.P.M. The volume of bacterial sediment was noted, after which the sediment was resuspended in sufficient physiological saline to make a final 1 per cent by volume suspension. Part of the suspension was removed to use as living vaccine, while the

remainder was heated at 57°C. for 30 minutes in sealed tubes submerged in a water bath. All heated suspensions proved sterile when planted on media capable of supporting the growth of *H. influenzae suis*.

Two of the strains used, 18 and 23, had been under cultivation sufficiently long that, while still capable of producing influenza when given intranasally to swine in mixture with swine influenza virus, they no longer transferred with the virus from sick to normal animals by contact (4). Strain 28 on the other hand had been but recently isolated and, with the virus, transferred readily from swine to swine by pen contact.

Source of Experimental Swine Employed.—Ordinarily, swine reared on the Institute farm are employed in experimental work. However, at the time that the present experiments were being conducted the supply of swine of our own rearing was limited making it necessary to purchase outside animals for use. 27 of these were obtained from a breeder in whose swine drove swine influenza had never appeared, to his knowledge. In a preliminary experiment 2 of these animals were tested for susceptibility to swine influenza by intranasal inoculation with a mixture of swine influenza virus and the bacterium *H. influenzae suis* and found to be fully susceptible. The remainder were bled, and samples of their blood sera tested for the presence of neutralizing antibodies for swine influenza virus. In a serum dilution of 1:2, three serum samples were found capable of partially neutralizing swine influenza virus. These three sera contained sufficient antibody to protect mice against death but not against the production of lesions when the usual neutralization technique was employed (5). This type of finding was different from what might have been expected had the antibodies arisen as the result of previous swine influenza infection; for serum from swine recovered from an attack of swine influenza neutralizes the virus completely in quite high dilution. At the time, the finding of partially neutralizing antibody in the sera of 3 of the animals was tentatively relegated to the vague classification of natural antibody, and the 3 animals supplying the sera were used in experiments other than those under discussion. The remaining swine in the group purchased were considered, on the basis of absence of neutralizing antibody in their sera and the full susceptibility of representative members of the group, not to have had previous experience with swine influenza and to be satisfactory for use in swine influenza experiments. The animals were about 2 months of age when purchased and were kept under observation in semi-isolation for almost 2 months prior to their introduction into the present experiments. They were all found to be infected in varying degrees with ascaris and lungworms, parasites which from past experience were not considered to influence materially the course of a swine influenza infection.

Attempted Vaccination of Swine with Heated and Living H. influenzae suis.—During December of 1936 each of 4 swine was given three intramuscular injections at 8 day intervals of heat-killed *H. influenzae suis*; a second group of 4 swine received injections similarly of living *H. influenzae suis*. The amount of the first dose administered was 1 cc., while the two succeeding doses were 2 cc. each. No noteworthy reaction was observed in any of the 8 swine following either their first or second injections of vaccine. However, after the third injection, in the cases of all 8 animals, a surprising and puzzling reaction

occurred. Since its character varied depending upon whether the animals had received living or heat-killed vaccine, the two groups will be discussed separately.

Reaction in Swine Vaccinated with Living H. influenzae suis.—On the 2nd day after the third injection, the temperature of swine 1843 rose to 40.9°C. and the animal appeared ill. The following day the animal was prostrated and had labored breathing. By the next day it appeared extremely ill, and it was moribund on the following day. It died on the 4th day after its initial temperature rise and the findings at autopsy were strongly suggestive of fatal swine influenza. 2 other animals, swine 1840 and 1847, exhibited temperature elevations to 40.9° and 40.4°C., respectively, on the 3rd day after their third injections of living *H. influenzae suis*. Swine 1840 was ill for 6 days with what clinically could not be distinguished from swine influenza. The illness of swine 1847 clinically resembled mild swine influenza and lasted for 4 days. The 4th animal in the group, swine 1844, developed a temperature of 41.2°C. on the 4th day after its third injection of *H. influenzae suis* and exhibited for 5 days an illness that was clinically indistinguishable from swine influenza.

Reaction in Swine Vaccinated with Heat-Killed H. influenzae suis.—All 4 of the swine injected with heat-killed *H. influenzae suis* exhibited an extremely mild and indefinite illness for 2 or 3 days, beginning on the 2nd or 3rd day after their third injection. The clinical picture shown by these 4 animals was characteristic of that seen in "filtrate disease" (1) and would probably have entirely escaped notice had not the 4 swine receiving the living *H. influenzae suis* vaccine been ill at the same time.

Experiments to Determine the Cause of the Disease Resulting from Multiple Injections of H. influenzae suis.—Pieces of lung of swine 1843, the animal which had died on the 4th day, were tested for the presence of swine influenza virus by mouse inoculation (6). An agent typical in all respects of swine influenza virus was demonstrated. Blood serum was obtained from the remaining 7 swine following their recovery, and all seven samples neutralized swine influenza virus completely, although failing to exert any effect on the PR8 strain of human influenza virus. Furthermore, the 7 recovered swine were subsequently tested for immunity to swine influenza and found to be fully immune. It thus seemed clear that the reaction observed in all 8 of the experimental animals following their third injection of *H. influenzae suis* had as its basis infection with the swine influenza virus. The disease observed in the animals injected with living *H. influenzae suis* was true swine influenza in that both the virus and the bacterial component were active; while the disease developing in the animals inoculated with heat-killed *H. influenzae suis* was "filtrate disease," such as is caused by experimental infection with the swine influenza virus alone (1), and apparently precipitated in the present instance by the inoculation with heated *H. influenzae suis*. No explanation of the source of the swine influenza virus responsible for these infections was apparent from consideration of the experiments just discussed.

Confirmation of the Findings

Late in January of 1937 4 more swine were placed in isolation and injected intramuscularly, as in the preceding experiments, with 1 per cent suspensions of heat-killed *H. influenzae suis*. On the 3rd day following the second injection this time, 2 of the 4 animals developed temperatures in the neighborhood of 41°C. and appeared mildly ill. The other 2 animals appeared mildly ill also, but their temperatures remained within normal limits. One of the febrile swine was killed on the 2nd day of fever and the other one on the 3rd day of fever, and at autopsy the findings in the respiratory tract were characteristic of a filtrate disease more extensive than usual. However, the lesions, instead of being limited to the anterior lobes as is usual in swine infected intranasally with virus, were diffusely scattered throughout the lung and were especially numerous at the bases of the diaphragmatic lobes. Swine influenza virus typical in all respects was demonstrated in both respiratory tracts by mouse inoculation. The 2 afebrile swine were kept under observation. They remained mildly ill for 2 days. They were bled 11 days later, and the serum of each neutralized swine influenza virus completely but was without effect on the PR8 strain of human influenza virus. It seemed clear that the reactions following the second injection in this group of experiments had been due to infection with the swine influenza virus. They thus confirmed the previous observations. The clinical picture exhibited by 2 of the animals was characteristic of filtrate disease; while in the remaining 2 which developed febrile reactions of 41°C. the clinical pictures were more severe than is ordinarily seen in swine infected with virus alone. The characteristics of the findings presented at autopsy were, however, typical of an extensive filtrate disease.

With this confirmation of the original observations it seemed that a regularly reproducible phenomenon was being dealt with. The situation, as it appeared from the data available at the time, could be summarized as follows: Apparently normal swine, given multiple intramuscular injections of suspensions of living *H. influenzae suis*, developed typical swine influenza in which both *H. influenzae suis* and swine influenza virus participated as infective agents. Similar swine given multiple intramuscular injections of heat-killed *H. influenzae suis* developed filtrate disease, in which the swine influenza virus was the sole infective agent. In neither set of experiments had swine influenza virus knowingly been introduced, and the origin of the virus infecting the swine was obscure.

Possible Sources of Virus

At the time, four possible sources of the virus were considered, either to be studied further or discarded as impossibilities. These may be briefly summarized as follows.

1. The virus might have been present as a contaminant of one of the cultures of *H. influenzae suis* used. This possibility could be eliminated on three grounds. First, direct test of the cultures by the intranasal inoculation of swine or mice failed to reveal virus; second, the heat-killed bacterial sus-

pensions had been heated well above the thermal death point of the virus; and lastly, had virus been present in the bacterial suspensions it should have immunized swine when given intramuscularly rather than induced infection (3).

2. The isolation technique might have been inadequate to prevent accidental infection. This possibility did not seem to furnish a reasonable explanation because at the time the experiments under discussion were conducted there were no cases of swine influenza in the laboratory. Furthermore, the isolation technique employed was the same as that used here for 8 years of more or less continuous investigation of swine influenza without an accidental cross-infection.

3. The swine used may have been carriers of swine influenza virus. This possibility was not considered very likely, because at the time no way of introducing swine influenza virus into swine was known that did not cause either infection or the acquisition of immunity. It had been established that virus given intranasally induced infection regularly, while administered by any other route it regularly immunized without causing recognizable infection. Since the swine used in the present experiments proved fully susceptible to infection and their sera were free of neutralizing antibodies, it had been concluded that they had not had a previous experience with swine influenza virus and thus could not be carriers of the virus. The possibility that virus might have gained access to the swine without either infecting or immunizing seemed remote.

4. The virus may have arisen *de novo* as a result of the experimental procedures to which the swine had been submitted. This possibility was included to be considered seriously only in case one of the three preceding was not found applicable.

Attempts to Extend the Observation and to Determine the Nature of the Phenomenon

Further experiments of the type described earlier were carried out in the hope of learning more of the phenomenon and determining the source of the swine influenza virus responsible for the infection that followed multiple injections of *H. influenzae suis*. At this phase of the investigation swine of our own rearing were again available and the supply of those purchased outside and used in the original experiments had been exhausted. Consequently in subsequent experiments our own swine were used. The first of these experiments failed completely to duplicate the original observation. So did the second and the third groups of experiments. Swine were given multiple intramuscular injections at 8 day intervals but remained perfectly normal throughout, neither acquiring swine influenza nor developing antibodies neutralizing swine influenza virus in their sera. As a result of this group of unsuccessful experiments the possibility was considered that the phenomenon

might be more closely related to the source of swine than had been considered likely in the beginning. Because of this, 8 more swine were purchased from the outside breeder who had furnished the original animals. These were of the same stock as purchased before but from later farrowings. After determining that their blood sera were free of swine influenza virus-neutralizing antibodies they were given multiple intramuscular injections of *H. influenzae suis*. No illness resulted from a long continued course of injections at 8 day intervals, nor did the animals develop swine influenza virus-neutralizing antibodies. With these failures it seemed apparent that a new attack on the problem was indicated.

Consideration of the experimental factors which might have changed between the time of the earlier positive experiments and the current negative ones suggested *H. influenzae suis* itself as probably the most labile. Because of the possibility that the cultures employed might have varied it was decided to obtain some fresh field strains for use. Seven strains were isolated in Iowa from naturally occurring cases of swine influenza. These seven cultures were pooled and administered intramuscularly to swine at 8 day intervals; but they, too, failed to induce a swine influenza virus infection in the experimental animals.

With the apparent exhaustion of the possibility that the source of swine or the cultures of *H. influenzae suis* themselves were responsible for the failure to duplicate the original experiments, other possibilities were considered. The original swine had been kept, prior to experimental use, in rather crowded quarters in a pen indoors, and it seemed that this fact might conceivably furnish a clue to the character of their peculiar reactivity to multiple injections of *H. influenzae suis*. Because of the crowding, cleaning of the pens had not been as scrupulous as it might have been under less crowded conditions, and it was reasoned from this that more than the usual opportunity had been afforded for the building up of heavy parasitic infections. It was furthermore reasoned, on the possibility that virus might have been made to arise *de novo*, that it would probably have been generated at the intramuscular site of injection of *H. influenzae suis*, under which circumstance it would have had to be transported in some way to the susceptible tissues of the respiratory tract. It seemed possible that the failure of the later experiments might have been due to a lack of this hypothetical transporting agent. Because the swine ascaris fitted the picture of a parasite whose larval stage migrated widely throughout the body before eventually becoming established in the gastrointestinal tract, experiments were planned in which wandering ascaris larvae would be present in the animals at the time of their second or third injections of *H. influenzae suis*. To this end, swine were fed embryonated swine ascaris ova (7) 2 or 3 days prior to their second or third injections of *H. influenzae*

suis.¹ Usually on the 8th day, occasionally somewhat later, after the ascaris feeding, the animals exhibited clinical signs of respiratory tract involvement. They became depressed, their respiratory rates were accelerated, and their temperatures were elevated to fever level. However, at autopsy the findings in the lung were only those characteristic of an ascaris pneumonia, and swine influenza virus could not be demonstrated in the respiratory tracts. Furthermore, swine that had been treated in this way and allowed to recover failed to develop swine influenza virus-neutralizing antibodies in their blood sera upon recovery. It thus seemed evident that the ascaris infestation had not furnished the requisite factor.

A number of other things were tried. Swine were kept in dirty pens, others were underfed, some were kept in cold isolation units, and others were kept in unusually warm isolation units; but under none of these conditions did multiple injections of *H. influenzae suis* exert the slightest effect so far as inducing a swine influenza virus infection was concerned.

In an accompanying paper experiments which explain the phenomenon will be reported.

SUMMARY

Multiple intramuscular injections of *H. influenzae suis* were found to precipitate swine influenza virus infections in a group of apparently normal swine. The most likely explanation of the phenomenon seemed to be that the animals, though healthy and susceptible, harbored the virus in some unknown manner. The factors possibly determining the phenomenon were explored experimentally but without success.

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¹ I am indebted to Dr. Norman R. Stoll and Dr. G. L. Graham for the advice and help they gave me in handling the parasitological aspects of these experiments.

THE SWINE LUNGWORM AS A RESERVOIR AND INTERMEDIATE HOST FOR SWINE INFLUENZA VIRUS

II. THE TRANSMISSION OF SWINE INFLUENZA VIRUS BY THE SWINE LUNGWORM

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In the preceding paper (1) experiments have been described in which swine influenza virus infections were elicited in apparently normal swine by multiple intramuscular injections of suspensions of either living or heat-killed *Hemophilus influenzae suis*. The findings suggested that the virus of swine influenza had been present somewhere in the pigs at the time of injection. In the present paper it will be shown that the swine lungworm is capable of harboring swine influenza virus and of transmitting it from swine to swine. This discovery was made incidentally to observations on the possible rôle of the lungworm in transmitting hog cholera.

Study of the possibility that the swine lungworm might serve as an intermediate host for hog cholera had as its basis the popular belief held by certain farmers and veterinarians in the Middle West that the earthworm is responsible, in some unvisualized fashion, for the persistence of cholera virus from one outbreak of the disease to the next.¹ The observations of the Hobmaiers (2) and Schwartz and Alicata (3) that the earthworm serves as intermediate host for the swine lungworm lent some plausibility to this belief, although it seemed more likely, if the earthworm were involved, that its rôle was indirect and that the lungworm in all probability was the actual carrier of the infectious agent. It was decided to test the possibility experimentally. In the hope that findings with one agent might serve as controls for those with another, a study with the swine influenza virus was conducted in parallel with that of the hog cholera virus. The latter yielded negative results whereas that with the influenza virus clearly showed that the lungworm was capable of serving as the transmitting intermediate host. For this reason the findings with the influenza virus will be especially stressed.

¹I am indebted to Dr. Fred J. Crow and Mr. Ivan Hummer for first calling my attention to this belief.

Materials and Methods

Viruses.—The swine influenza virus used was strain 15 obtained originally in Iowa in 1930 from a naturally occurring case of swine influenza. It had been maintained by passage through swine at intervals of 2 months or less.

The strain of hog cholera virus employed had been obtained from a commercial laboratory in 1937. Prior to use in the present experiments it had been maintained by occasional passage through swine at this laboratory.

Earthworms.—The earthworms were dug locally in areas known to have been free of contamination by swine feces for at least 10 years. Furthermore, repeated examinations of representative specimens of earthworms from these areas have failed to reveal the presence of swine lungworm larvae. Four species of earthworms,² *Eisenia foetida* (Savigny), *Allolobophora caliginosa* f. *typica* (Savigny), *Allolobophora longa* Ude, and *Lumbricus terrestris* Linnaeus, have been utilized in the experiments, and all seem equally capable of serving as intermediate hosts for the swine lungworm.

The earthworms for each experiment were kept in separate wooden barrels of the type in which bulk chemicals are shipped. These barrels, measuring approximately 18 inches in diameter by 30 inches in depth, were buried in the earth to within 2 or 3 inches of their tops and filled with soil to a level which eventually approximated that of the outside dirt level. Each barrel was covered with a fairly snugly fitting wooden lid. Food for the experimental worms consisted of a handful of either yellow corn meal or used coffee grounds, scattered on the dirt surface of the barrels at intervals of 6 weeks to 2 months, and a 2 to 3 inch surface layer of decaying maple and linden leaves which was maintained at all times. This leaf mulch, in addition to furnishing nutriment, also served to maintain the correct degree of moisture in the upper portion of the dirt. Water was sprinkled on the dirt surface at irregular intervals, depending upon weather and season, sufficiently often to maintain the soil in a moist but not wet condition.

Lungworms.—In the present experiments two species of lungworms,³ *Metastrongylus elongatus* and *Choerostongylus pudendotectus*, have been used in mixture, and no attempt has yet been made to work with either species singly. The developmental cycles of these two lungworms are the same. They are concurrently present in swine of the Institute herd having free access to pasture.

The Lungworm Cycle in Earthworms

The swine lungworm, a nematode parasitic in the bronchioles of the bases of the lungs of swine, has been shown by the Hobmaiers (2) and Schwartz and Alicata (3) to pass the first three of its developmental stages in an earthworm. The life cycle in brief is as follows. The embryonated lungworm ovum deposited in the swine respiratory tract by the female lungworm is coughed up, swallowed, and eventually passed in the swine feces. After reaching the soil it is swallowed by an earthworm, in which it hatches as a first-stage larva. It undergoes two further developmental

² I am indebted to Dr. Libbie Hyman, Dr. Grace Pickford, and Dr. Henry Olson for the identifications of the earthworms used.

³ I am indebted to Dr. Norman R. Stoll for identifying the lungworm species used.

stages in the earthworm eventually reaching its third or infective larval stage. In this stage it is capable of infesting swine and has usually become localized either



FIG. 1. Third-stage lungworm larvae as seen in a fresh "press" preparation of the calciferous gland of an experimentally infested earthworm. $\times 94$. Photographed by Mr. Julian A. Carile.

in the calciferous glands, hearts, or gizzard of its earthworm intermediate host. Examination of fresh "press" preparations of these organs of an infected earthworm under the low power of the microscope readily reveals the presence of larvae (Fig. 1). The larva remains in the third stage until its earthworm host is ingested by a swine. Once within the swine's gastrointestinal tract the larva is liberated, penetrates the swine

intestinal mucosa, and migrates to the respiratory tract by way of the lymphatics and blood stream. It undergoes two further developmental stages in the swine, finally becoming an adult lungworm in the bronchioles at the bases of the diaphragmatic lobes. The whole of the cycle can occupy a span of several years for its completion or, under the most favorable conditions, can be completed in about 2 months.

It is the third larval stage which permits delay in completion of the developmental cycle. In this stage the larva can apparently persist for periods of at least 4 years (4) in its earthworm host.⁴ It is probably because of this very favorable and prolonged survival period in an intermediate host that lungworms constitute such a common parasite in swine reared under the usual farm conditions.

Preliminary Tests

Swine Influenza.—Late in October of 1938 lungworms were obtained at autopsy from 3 swine killed on the 3rd, 4th, and 5th days after infection with swine influenza (intranasal inoculation with a mixture of the bacterium *H. influenzae suis* and swine influenza virus) (5). The worms, in a Petri dish containing a little water, were minced coarsely with scissors to release the ova they contained. This worm mince was then buried 3 to 4 inches below the dirt surface in a sunken barrel. Feces collected from the same 3 swine throughout the course of their illnesses together with the colon feces obtained at autopsy were similarly buried in the barrel and loosely mixed with dirt. Shortly afterwards about 400 earthworms were placed on the surface of the soil in the barrel and the dirt was thoroughly wet with a sprinkler of water. The worms promptly burrowed beneath the surface. This barrel was designated as No. 4.

Five weeks later representative earthworms were examined and in their calciferous glands were found third-stage lungworm larvae ranging in numbers from single specimens to as many as 20 or more.

After the lungworm larvae had become established in their earthworm intermediate hosts an attempt was made to learn whether these larvae, hatched from ova obtained from influenza-infected swine, could induce influenza when they infested normal swine. 50 earthworms were removed from the barrel and, after thorough washing in tap water, were fed to 2 swine. In order to be certain that all of the earthworms were consumed they were cut into lengths of a centimeter or less and mixed with the ground grain feed of the swine. The pigs ate this worm-grain mixture with apparent relish.

Hog Cholera.—The hog cholera experiment paralleled that with swine influenza both chronologically and in methods used. The lungworms and feces containing lungworm ova were obtained from 2 swine killed when moribund of hog cholera on the 6th and 10th days after infection with hog cholera virus. This material was fed to a similar number of earthworms in another sunken barrel which was designated as No. 3. These earthworms became infested with larval lungworms in numbers com-

⁴The life span of the earthworm itself, under natural conditions, is unknown. However, under the artificial conditions of captivity specimens of *E. foetida* have lived for as long as 4½ years, *L. terrestris* for as long as 6 years, and *A. longa* for as long as 10¼ years. (Stephenson, J., *The Oligochaeta*, Oxford, The Clarendon Press, 1930, 637.)

parable to the earthworms in the influenza barrel and at 5 weeks 50 were dug and fed to 2 swine as in the influenza experiment.

The 2 swine used in each experiment were normal so far as any past experience with either the hog cholera or the swine influenza virus was concerned. However, all 4 animals, for 3 weeks prior to being employed in the earthworm experiments, had received a series of 3 intramuscular inoculations of suspensions of the bacterium *H. influenzae suis* at 8 day intervals. These inoculations had caused no clinical illness in any of the 4 swine, nor was there any reason to suppose that they had altered the susceptibility of the swine to either hog cholera or swine influenza virus. The animals were considered "normal" with respect to the experiments in which they were to be used. However, as later developments demonstrated, the preliminary inoculations with the bacterium *H. influenzae suis* were of particular significance in determining the results obtained.

Swine 2149 and 2215, that had been fed earthworms containing lungworm larvae from hog cholera animals, and swine 2162 and 2217, fed earthworms containing lungworm larvae from swine influenza animals, were observed for a period of 10 days. All remained normal, and the outcome of the experiments was believed to be negative. Rather than destroy the animals at this time it was decided to continue their courses of intramuscular inoculations of suspensions of the bacterium *H. influenzae suis* that had been interrupted at the time of their introduction into the lungworm experiments. Consequently on the 10th day after they had been fed earthworms each swine was inoculated intramuscularly with 2 cc. of a 1 per cent by volume suspension of a 22 hour culture of *H. influenzae suis* grown on potato-chocolate agar slants. The 2 cholera-worm animals, swine 2149 and 2215, remained normal throughout a further period of observation of 13 days.

As has been reported briefly in a preliminary publication (6) the 2 influenza-worm animals came down with swine influenza.

On the 3rd day after its intramuscular inoculation with *H. influenzae suis* swine 2217 developed a temperature of 40.9°C. and appeared ill (Fig. 2). The following day its temperature was still elevated and the clinical signs exhibited were those characteristically seen in swine influenza. The animal remained febrile and ill for 4 days and then underwent an uneventful recovery. Swine 2162 in the same pen remained normal until 5 days after swine 2217 had first become sick and then it too underwent a 4 day illness that was clinically characteristic of mild swine influenza. Serum drawn from each of these 2 animals during convalescence was found to contain antibodies neutralizing swine influenza virus, whereas that drawn prior to the earthworm feeding had been devoid of antibodies. The serum of neither of the swine fed the cholera-worms developed antibodies neutralizing swine influenza virus during a similar period of observation.

It seemed apparent from these experiments that the 2 swine fed lungworm larvae from pigs with swine influenza had undergone attacks of typical swine

influenza. However, in the light of subsequent experiments of this type it is probable that only swine 2217 acquired its swine influenza virus directly from the lungworm larvae ingested. The illness of the other animal in the same pen, swine 2162, probably represented an infection acquired by contact with swine 2217. Furthermore the experiments suggested that more than mere transfer of virus by infected lungworm larvae was required to elicit infection: A provocative stimulus or stress was also essential. In the above experiment intramuscular injections of *H. influenzae suis* had provided the provocation.

So far as the single experiment with lungworm larvae from cases of hog cholera was indicative, it did not appear that the hog cholera virus was capable of transmission *via* the lungworm. Because of the promising lead obtained with swine influenza it was decided to concentrate on this phase of the problem and to abandon, for the time, further investigation of hog cholera.

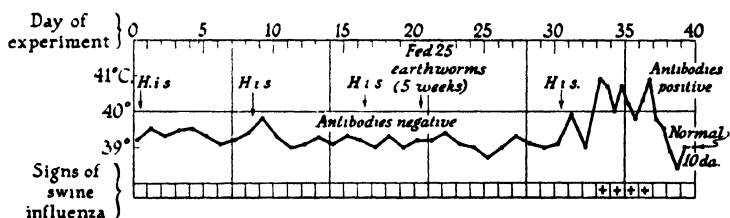


FIG. 2. Swine 2217. The animal received three intramuscular injections of *H. influenzae suis* (H. i. s.) before being fed 25 earthworms 5 weeks after these had ingested lungworm ova from swine with swine influenza. Afterwards a fourth injection of *H. influenzae suis* was followed by clinically characteristic influenza. During convalescence virus-neutralizing antibodies appeared in the animal's serum.

Results of Confirmatory Experiments

Experiment 1.—In January of 1939, 2 swine, 2200 and 2291, were each fed 18 earthworms removed from barrel 4. By now 2 months had elapsed since the first exposure of these earthworms to lungworm ova from pigs with swine influenza. 19 days after being fed the earthworms each swine was inoculated intramuscularly in the ham with 1 cc. of a 1 per cent by volume suspension of a 48 hour potato-chocolate agar live culture of *H. influenzae suis*. The animals remained normal. 8 days later each was given a second intramuscular injection of 2 cc. of a 1 per cent suspension of live *H. influenzae suis*.

On the 4th day after this second injection the temperatures of both animals rose abruptly and on the following day they exhibited clinical signs characteristic of swine influenza (Fig. 3). Swine 2200 was killed and autopsied on the 3rd day of illness. At autopsy the pathological alterations in the lung were characteristic of those of swine influenza, and swine influenza virus was demonstrated in the lung by mouse inoculation (7, 8). The distribution of the lesions in swine 2200 was somewhat different, however, than that ordinarily seen in intranasally inoculated swine (9). In-

stead of the pneumonia being limited to the cephalic and cardiac lobes as is usually the case, it was rather diffusely distributed in a lobular fashion and portions of all lobes were involved. From past experience with swine infected nasally, extensive involvement of the diaphragmatic lobes except in fatal cases had come to be looked upon as very exceptional. The pneumonia at the extreme bases, in the regions of bronchioles containing adult lungworms, was especially marked.

The other animal, swine 2291, was ill for 3 days and then underwent an uneventful recovery. Its blood serum, obtained after recovery, was found to have developed antibodies neutralizing swine influenza virus.

The demonstration of swine influenza virus in the respiratory tract of one animal during the acute stage of illness and of specific virus-neutralizing antibodies in the blood serum of the other after recovery made it evident that the influenza-like illness of each had indeed been swine influenza and had had as

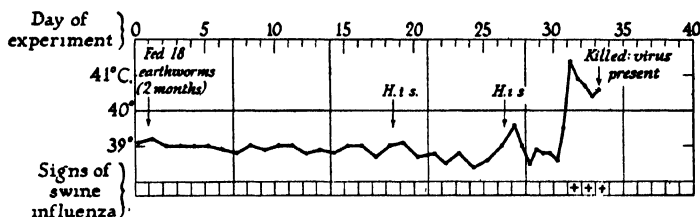


FIG. 3. Swine 2200 was fed 18 earthworms which 2 months previously had ingested lungworm ova from swine with swine influenza. 19 and 27 days later the swine was injected intramuscularly with *H. influenzae suis* (H. i. s.). 4 days after the second injection the animal developed swine influenza that was characteristic clinically and at autopsy. Swine influenza virus was demonstrated in the respiratory tract.

its basis infection with the swine influenza virus. The bacterium *H. influenzae suis* was present in cultures from the trachea of the first animal.

Experiment 2.—Late in January of 1939 swine 2222 and 2240 were given two intramuscular injections of live *H. influenzae suis* at an 8 day interval. 3 days after the second injection each animal was fed 7 earthworms removed from barrel 4. By now 3 months had elapsed since the first exposure of these earthworms to lungworm ova from pigs with swine influenza. The animals remained normal throughout an 18 day period of observation. Then each was inoculated intramuscularly in the ham with 2 cc. of a 1 per cent suspension of live *H. influenzae suis*.

On the 3rd day after this inoculation the temperature of swine 2222 rose abruptly and the animal appeared ill (Fig. 4). The following day its temperature had risen to 41.3°C. and the clinical signs were those characteristic of swine influenza. The animal was killed and autopsied and the findings in the respiratory tract were typical of those seen in swine influenza. As in the animal autopsied in the preceding experiment, however, the distribution of the pneumonia tended to be more basilar than ordinarily encountered in swine experimentally infected with swine influenza by the

nasal route. Swine influenza virus was demonstrated in the pneumonic lung of this animal, and in lungworms taken from bronchi at the bases, by mouse inoculation. *H. influenzae suis* was present in cultures from the respiratory tract.

Swine 2240 became ill on the 6th day after inoculation and the findings at autopsy on the 3rd day of illness were similar to those described for swine 2222. Swine influenza virus was demonstrated in the lung and in lungworms from this animal by mouse inoculation. *H. influenzae suis* was present in cultures from the trachea. In the light of subsequent experience it is likely that swine 2240 acquired its infection by exposure to swine 2222, the first animal of the pair in the pen to sicken.

Experiment 3.—Late in April of 1939, 5 swine, 2345, 2340, 2339, 2344, and 2341, that had been reared indoors on a concrete floor and were known to be free of lungworms were each fed 15 earthworms from barrel 4 (5 earthworms on each of 3 consecutive days). 6 months had now elapsed since these earthworms had ingested their

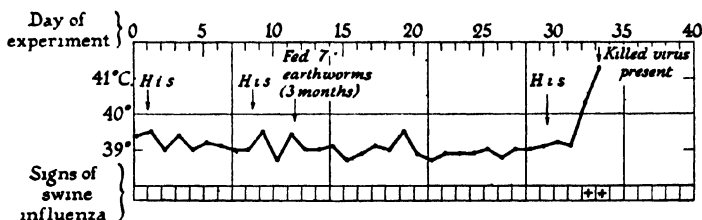


FIG. 4. Swine 2222 received two intramuscular injections of *H. influenzae suis* (*H. i. s.*) before being fed 7 earthworms which 3 months previously had ingested lungworm ova from swine with swine influenza. The animal remained normal for 18 days and was then again injected intramuscularly with a suspension of *H. influenzae suis*. On the 3rd day after this injection it came down with swine influenza. Virus was demonstrated in its respiratory tract.

lungworm ova. Swine 2340 was subsequently given three intramuscular injections of suspensions of live *H. influenzae suis* at 8 day intervals beginning 13 days after the earthworm feeding. It failed to become ill and was killed and autopsied. No lungworms could be found upon careful search of its respiratory tract. Another of the group, swine 2345, was then killed and autopsied, and its respiratory tract also proved free of lungworms. It was thus apparent that lungworms had for some unknown reason failed to become established in this group of swine. Consequently in June swine 2339 and 2344 were again fed 18, and swine 2341, 6 earthworms, from barrel 4. Throughout the remainder of the summer they were subjected to inoculations with suspensions of *H. influenzae suis* at irregular intervals, but failed to become ill. Swine 2344 was finally killed and autopsied late in July. Except for the presence of moderate numbers of lungworms in the bronchi at the bases of its diaphragmatic lobes its respiratory tract was normal. Furthermore, blood sera from all 3 swine at this time were devoid of virus-neutralizing antibodies. Swine 2339 and 2341 were kept under observation and received no further inoculations of *H. influenzae suis* until Aug. 18. Then each received a series of three intramuscular injections at 8 day intervals.

On the 3rd day after the third injection of *H. influenzae suis* the temperature of swine 2341 rose abruptly to 40.9°C. and the animal appeared ill. The following day the clinical signs were those characteristic of a mild swine influenza. By the next day the temperature had dropped to normal and the animal appeared to be recovering. It was killed and autopsied and the pathological findings in the respiratory tract were those of swine influenza. Again, however, the pneumonia was predominantly basilar instead of being limited to the anterior lobes as is usual in swine infected by way of the nose. Lungworms were numerous in the bronchi at the bases of the lung.

Eight anesthetized mice were inoculated intranasally in the usual fashion (8) with a 10 per cent suspension of pneumonic lung of swine 2341 in order to test for the presence of swine influenza virus. 4 of these were killed and autopsied on the 4th day. Their lungs appeared completely normal. The lungs of these mice were ground in saline to make a 5 per cent suspension and this suspension was administered intranasally to 8 more anesthetized mice. Of these, 5 died and 3 were killed, and all showed lung lesions characteristic of those caused by swine influenza virus. Furthermore in the next serial passage, mice which received a suspension of lungs of these 2nd passage mice mixed with serum known to neutralize swine influenza virus were completely protected, whereas the control mice all died. The remaining 4 mice that had received the original lung suspension from swine 2341 were tested for immunity to a fully mouse-adapted swine influenza virus 25 days later. One of these died and the other 3 survived.

It seemed apparent from these experiments that the lung of swine 2341 had contained swine influenza virus. The failure of this virus to kill, as most swine influenza viruses typically do, in its first mouse passage, suggested that its mouse pathogenicity may have been altered in some way either by its sojourn of 8 months in lungworm larvae in their earthworm intermediate hosts or its survival of almost 3 months in the respiratory tract of swine 2341 prior to causing illness.

The remaining animal in Experiment 3, swine 2339, developed swine influenza 2 days after swine 2341 became ill in the same pen. It is believed that this constituted a contact infection. This animal was only moderately ill. Its blood serum, obtained 10 days after infection, neutralized swine influenza virus.

Experiment 4.—In October of 1939 swine 2428 and 2432, known to be free of lungworms, were each fed 12 earthworms from barrel 4 (4 earthworms on each of 3 consecutive days). It had now been a year since these earthworms had ingested their lungworm ova. Beginning 14 days after their earthworm feeding each animal received four intramuscular injections of suspensions of live *H. influenzae suis* at 8 day intervals. The first of these injections in each case was 1 cc. of a 1 per cent suspension of 48 hour potato-chocolate agar cultures, while subsequent injections were of 2 cc. Midway between the third and fourth *H. influenzae suis* inoculation each animal had received 10 cc. of a 5 per cent solution of calcium chloride into the right lung and

pleura through the chest wall, a procedure which was known, from experiments to be reported later, to elicit swine influenza virus infections sometimes in swine that had ingested lungworm larvae from influenza swine. The use of calcium chloride to provoke swine influenza virus infections had been suggested originally by the finding of Bullock and Cramer (10) that this substance would break the dormancy of spores of the bacteria of gas gangrene and tetanus in mice or guinea pigs.

Neither animal was noted to be clinically ill as a result of any of the procedures to which it had been subjected and the experiments were considered negative. Swine 2432 was killed and autopsied 5 days after its last injection of *H. influenzae suis*. Its respiratory tract was negative except for the presence of dense fibrous adhesions in the right pleura resulting from the calcium chloride injection. There were also numerous adult lungworms in the bronchi of the bases of the diaphragmatic lobes.

Blood serum obtained at autopsy was saved to test for the possible presence of antibodies neutralizing swine influenza virus, a procedure regularly followed in this work. Surprisingly enough in view of the fact that the animal had at no time been clinically ill, its serum neutralized swine influenza virus to a dilution of 1 in 8. This titer was lower than that ordinarily encountered in the sera of convalescent swine (11). However, since the serum of the animal drawn 6 days after its earthworm feeding was free of virus-neutralizing antibodies the presence of antibodies later on must be interpreted as having resulted from an experience with the swine influenza virus.

Since this experience did not result in clinically recognizable illness there is no way of knowing which of the several procedures applied to the animal had elicited the virus response.

The other animal in the pen, swine 2428, failed to become ill, nor did virus-neutralizing antibodies appear in its blood serum in spite of the continuation of efforts to elicit a swine influenza infection by multiple intramuscular inoculations of *H. influenzae suis*. When this animal was finally killed and autopsied its respiratory tract was normal except for the presence of numerous adult lungworms in the bronchi of the bases of the diaphragmatic lobes.

In this experiment, then, not only did swine 2432 undergo an immune response to swine influenza virus, but it failed to transmit its virus by contact to swine 2428 in the same pen. The failure of swine influenza virus to transfer by pen contact is of exceedingly rare occurrence in swine that have been infected nasally.

Experiment 5.—In March of 1940 swine 2433, known to be free of lungworms, was injected intramuscularly with 1 cc. of a 1 per cent suspension of a 48 hour potato-chocolate agar culture of *H. influenzae suis*. On each of the 2 succeeding days the animal was fed 5 earthworms from barrel 4. 16½ months had now elapsed since these earthworms had ingested lungworm ova from pigs with swine influenza. 11 and 19 days after the earthworm feeding the pig was inoculated intramuscularly with 2 cc. of a 1 per cent suspension of live *H. influenzae suis*. No illness resulted from either injection. 4 days later, because of a suggestion furnished by Taylor's (12) observa-

tion that sterile fluid into the respiratory tracts raised the influenza virus titer of sublethally infected mice, 15 cc. of broth was administered intratracheally under chloroform-ether anesthesia. This was followed in 2 days by the intramuscular injection of 1 cc. of a 1 per cent suspension of living *H. influenzae suis*.

On the 2nd day after this last injection the animal appeared ill, and its temperature rose abruptly to 41.1°C. It was killed and autopsied on this 1st day of illness. The findings at autopsy were unusual in that the cephalic, cardiac, and azygos lobes were free of lesions. There was, however, a scattered lobular atelectatic pneumonia of the upper portion of the right diaphragmatic lobe, and the bases of both diaphragmatic lobes were consolidated. There were numerous small but mature (embryonated ova present in the females) lungworms in the bronchi at the bases.

In this instance swine influenza virus of average pathogenicity for mice was demonstrated in the respiratory tract. In its first passage it killed all mice inoculated within 8 days and in the second mouse passage killed all within 5 days. The finding of virus that was fully pathogenic for mice in this pig eliminated from consideration the possibility that the apparent attenuation of the virus for mice in the case of swine 2341 (Experiment 3) had resulted from its 8 month sojourn in lungworm larvae. Here in the case of swine 2433 virus fully pathogenic for mice had been recovered from an animal infected with virus that had survived for 16½ months in lungworm larvae.

Swine 2433 transmitted influenza, by exposure, to another pig in the same pen, and antibodies neutralizing swine influenza virus appeared in the serum of this contact animal during convalescence.

Experiment 6.—In November of 1940 swine 2609 and 2629, known to be free of lungworms, were each fed 5, 6, and 10 earthworms from barrel 4 on 3 consecutive days. 2 years had now elapsed since these earthworms had ingested lungworm ova from pigs with swine influenza. Beginning 9 days after their earthworm feeding the pigs were given three intramuscular inoculations of suspensions of live *H. influenzae suis* at 8 day intervals.

No clinically recognizable illness resulted. However, blood serum obtained from both swine 22 days after their third inoculation with *H. influenzae suis* was found to neutralize swine influenza virus. The neutralizing titer of the serum was 1:20 in the case of 2609 and slightly less in the case of 2629 when tested against the usual 1000 M.L.D.'s of swine influenza virus. Serum of each swine obtained at the beginning of the experiment had been free of neutralizing antibodies. The serum antibody titer attained was thus lower than that ordinarily encountered in swine convalescent from actual infection (11). It more nearly approached that of swine immunized by virus administered subcutaneously or intramuscularly.

Because both swine remained clinically normal throughout the experiment it is not possible to know which of the provocative inoculations activated the virus. However, the antibody response indicated that each swine had undergone an experience with the swine influenza virus.

Failure to Demonstrate Swine Influenza Virus in Larval or Adult Lungworms

Consideration of the courses of the experiments just outlined and of the procedures required to elicit swine influenza infections made it evident that the swine influenza virus contained in the lungworms must not have been in a readily available or infective form. If fully infective virus were present within lungworm larvae one would anticipate that a swine influenza infection would result as soon as the larvae reached the highly susceptible tissues of the swine respiratory tract, that is to say on from the 3rd to the 8th day after ingestion. Since infections failed to occur then and infested swine remained normal for long periods of time, even after the larvae had developed to adult lungworms, it seemed obvious either that the virus in the worms was present in a masked, non-infective form or that it was not liberated from within the infected lungworm cells until some unusual stress was applied.

A number of experiments have been conducted in an attempt to demonstrate swine influenza virus within larval or adult lungworms by direct or indirect means. Since these have so far been uniformly negative only the more general aspects of the procedures tried will be outlined.

Efforts were first made to demonstrate virus in third-stage larvae.

Calciferous glands and hearts, rich in third-stage lungworm larvae, were removed from earthworms of known influenza-producing capability. These were ground with sterile sand, and suspended in saline. In such suspensions the larvae were thoroughly disintegrated. The suspensions were then administered intranasally to either 6 or 8 anesthetized mice in each experiment. Half of the mice were killed and autopsied on the 4th day after inoculation, and suspensions of their lungs were passed intranasally to a second group of anesthetized mice. This procedure was continued through either three or four serial passages before concluding that swine influenza virus was non-detectable.

In no case were pulmonary lesions, suggestive of those caused by swine influenza virus, observed. 2 or 3 weeks later, the surviving half of the mice through which the material had been passaged were inoculated intranasally with swine influenza virus. All succumbed typically, indicating that no immunity had been conferred by the lungworm larvae suspensions.

In addition suspensions of lungworm larvae similar to those administered to mice were mixed with cultures of *H. influenzae suis* and inoculated intranasally, intratracheally, or directly into the lungs of swine. The animals failed to come down with swine influenza nor did they develop in their sera antibodies neutralizing swine influenza virus. From such experiments it seemed apparent that virus in the lungworm larvae was present either in subinfective titer or in a non-infective or thoroughly masked form.

Efforts were next made to demonstrate virus in adult lungworms removed from the respiratory tracts of swine thought to be "ripe" for provocation of influenza.

The swine furnishing these lungworms had been fed earthworms just as in the experiments outlined earlier. However, they were not submitted to provocative inoculations with *H. influenzae suis* but instead were killed and autopsied a month or longer after their earthworm feeding. The adult lungworms contained in their respiratory tracts were removed, ground with sand, suspended in saline, and administered to mice and swine in a manner similar to that used in testing the larvae for virus.

Neither mice nor swine became infected or developed immunity, and it was necessary to conclude from the tests that swine influenza virus was not demonstrable in the adult lungworms.

An attempt was next made to demonstrate the presence of virus in adult lungworms from "ripe" swine by indirect means. Though the virus was masked and non-infective upon direct introduction into the respiratory tracts of susceptible hosts it yet might prove detectable by means that had been successfully used to demonstrate the presence of masked virus in another disease, rabbit papillomatosis. Here, although suspensions of the papillomas of domestic rabbits are usually non-infectious due to masking (13) of the virus they contain, they will immunize other rabbits to the virus and will even elicit low titer virus-neutralizing antibodies (14) if administered intraperitoneally.

Applying the same general procedure used in detecting masked papilloma virus, mice were given repeated intraperitoneal injections of suspensions of lungworms from "ripe" swine. 2 weeks after their last injection they were inoculated intranasally, while etherized, with from 100 to 1000 M.L.D.'s of swine influenza virus. All succumbed typically and no immunity was demonstrable.

It must be concluded from the experiments conducted so far that swine influenza virus is not detectable by either direct or indirect means in lungworms known from other evidence to be acting as intermediate hosts for the virus.

DISCUSSION

The experiments described were undertaken in an effort to learn whether the swine lungworm could serve as intermediate host for either the hog cholera or the swine influenza virus. It had been anticipated, if the lungworm were to fit the rôle of intermediate host for either virus tried, that it would transmit the causative agent from sick to normal animals directly. In view of this preconceived notion that the lungworm should fit the general pattern of other known intermediate hosts, the first experiment tried in the case of each virus was considered negative when, after a 10 day period of observation, the animals to which lungworms suspected of carrying virus had been administered, remained normal. 10 days was well beyond the usual incubation period for either hog cholera or swine influenza and furthermore within this period the lungworm larvae should have largely completed their migrations within the host and have become established in the swine respiratory tract. The pos-

sibility that either virus might have been transmitted in a latent, masked, or non-infective form was not considered until, in the course of subsequent events, the findings were such as to make that possibility quite obvious. Because the first experiment with hog cholera virus was negative, further work with it was discontinued and only the more promising experiments with swine influenza were carried further.

It was found that the suggestion furnished in the first swine influenza experiment, that virus transmitted by the lungworm had to be activated in some way to elicit infection, was indeed correct. Swine that had been fed earthworms containing lungworm larvae hatched from ova coming from influenza-infected swine remained normal to all appearances so long as they were kept under the usual experimental conditions. Only when they were subjected to a stress of some unusual character did they develop influenza. The administration of multiple intramuscular injections of suspensions of the bacterium *H. influenzae suis* constituted a satisfactory means of furnishing the required stress. It is probably significant that in no case did influenza follow a single injection of the bacterial suspension. Sometimes it followed the second or third injection, but not infrequently a larger series had to be employed. It is believed that these findings indicate that the provocative stimulus responsible for eliciting the swine influenza infections was not *H. influenzae suis per se* but rather some condition or chain of conditions established by repeated injection of the bacterium. Conceivably it partook of the character of an allergic or sensitization phenomenon.

After infection with the virus was provoked, however, the presence of *H. influenzae suis* was important in determining the character of the resulting disease. In all of the experiments in which clinical illness ensued, *H. influenzae suis* administered intramuscularly had reached the respiratory tract so that, upon activation of the virus, true swine influenza, having as its cause the concerted activity of *H. influenzae suis* and swine influenza virus (5), resulted. In some experiments in which many injections of *H. influenzae suis* had been administered before the virus infection was finally provoked, the resulting swine influenza was milder than usual, probably because of the development of some degree of immunity to *H. influenzae suis* (15). (The case of swine 2341, Experiment 3, illustrates this.) In other experiments which have been carried out but which will not be reported until later, provocation by means other than the use of live *H. influenzae suis* yields filtrate disease, an infection in which only the virus participates.

The experiments reported, all conducted with earthworms taken from barrel 4, give a false impression of the ease and regularity with which the phenomenon under discussion can be reproduced. To date, a total of 69 experiments, containing from 1 to 8 swine per experiment, have been conducted. Of these, 41 have either proved negative or been discontinued before infections were

provoked. The remaining 28 experiments, including those cited in the present paper, have been positive. There are numerous reasons for this only partial success. Undoubtedly some of the failures resulted from the use of lungworms that, for a variety of reasons to be discussed in a later paper, had not become carriers of swine influenza virus. In most of the negative experiments, however, the failures are believed to have been due to the inability to establish, in the experimental swine, appropriate conditions for the virus infection. Only one of the reasons for failures in this category is apparent from the experiments cited, and only this one will be discussed.

The swine in experiment 3 were fed late in April earthworms known to contain lungworm larvae. Because lungworms failed to become established the procedure was repeated in June. From then until September efforts to provoke influenza infections in these animals were unsuccessful. In September, after having proved refractory throughout the summer, one of the animals finally developed swine influenza following a long series of provocative inoculations. In other experiments not included among those dealt with in this paper a similar summer refractory state has been encountered. During 1939 no experiments were successful between April and September, while in 1940 all experiments carried out from May to October were negative. It would seem from such results during two summers that, if swine are not completely refractory during this time of the year, their infections are at least much more difficult to elicit using the same means of provoking that have been successfully used during the autumn, winter, and spring months. This failure of the virus to cause infection by way of its lungworm intermediate host during the summer has, as yet, no explanation. The finding, however, fits well with the known seasonal incidence of swine influenza under field conditions. The result obtained in Experiment 3 indicates, so far as it goes, that the refractory state is due not to the inability of the worm intermediate host to transmit its masked virus during the summer but rather to failure of the provocative stimuli applied to render the masked virus infective during this season of the year. While a number of the 41 negative experiments may have resulted in failure because they were conducted during the summer refractory state, others carried out during known favorable seasons have also resulted negatively. The question of the rôle played by seasonal and other factors in determining the transmission of swine influenza virus by way of a lungworm intermediate host will be considered in detail in a later paper when experiments other than those included in the present paper have been described.

The inability to detect swine influenza virus by direct means either in lungworm larvae in their earthworm intermediate hosts or in adult lungworms removed from the respiratory tracts of swine thought ripe for provocation of the disease has been an interesting but bothersome handicap to the work. While the non-infectiousness of the virus in its intermediate host was to be

anticipated from the failure of infected lungworms to induce disease directly in the swine whose respiratory tracts they infested, it had been hoped that there might be some way of demonstrating its presence by indirect means. Thus far all attempts to do this have been unsuccessful and it is necessary to conclude that the swine influenza virus is in a completely masked non-infective form in its lungworm intermediate host. It evidently remains in this form until unmasked by some stress applied either to the lungworm or to the swine harboring it. This phenomenon of provoking the infectivity of masked swine influenza virus is reminiscent of those experiments of Bullock and Cramer (10) in which the dormancy of spores of the bacteria of gas gangrene or of tetanus in mice or guinea pigs was broken by injecting calcium chloride, a phenomenon which was termed kataphylaxis. In like manner, Turner (16) has demonstrated in Black Disease of sheep that spores of *Bacillus oedematiens*, the causative bacterium, may be present in a latent state in the livers of sheep for long periods of time without causing disease. Only when a kataphylactic agent invades the liver, in this case the liver fluke *Fasciola hepatica*, does Black Disease ensue.

In order to demonstrate the presence of swine influenza virus in its lungworm intermediate host it is necessary that the lungworm go through its complete cycle beginning in the respiratory tract of an infected swine and ending in the respiratory tract of a susceptible and properly prepared swine. Fully infective virus is detectable only at either end of such a cycle. Stanley (17) has recently compared this phenomenon very aptly with that of a train passing through a tunnel: One can see the train as it enters and as it leaves, but it is no more apparent while in the tunnel than is swine influenza virus while in its intermediate host.

Nothing to indicate that swine influenza virus is injurious to its lungworm host has been observed. Neither is there any evidence that lungworms in the swine respiratory tract are injured by the provocative stimuli applied in eliciting virus infections.

These experiments with swine influenza are not the first instance in which it has proved difficult or impossible to detect the presence of an infectious agent during the time that it is in an intermediate or transmitting host, though it is, so far as is known, the first instance in the case of a filtrable virus. In salmon poisoning of dogs, the non-filtrable causative agent, whose nature remains obscure, is transmitted by the fluke *Nanophyetus salmincola*. Though the etiological agent is demonstrable, by dog inoculation, in the encysted cercariae of the fluke in its fish host it is not similarly detectable in the rediae or cercariae of the fluke in the snail host (18). Furthermore in blackhead of turkeys, a disease in which the causative histomonad (*Histomonas meleagridis*) is transmitted by the cecal worm *Heterakis gallinae*, histomonads have not been demonstrated by direct means in *Heterakis* ova (19). Stanley's tunnel simile is as

applicable to the histomonad of blackhead as it is to the virus of swine influenza. If agents having the character of a histomonad or of the non-filtrable agent responsible for salmon poisoning are not, by any available means, demonstrable in their intermediate hosts, it is perhaps not surprising that a filtrable virus should be "lost" in its transmitting host. The occult nature of infectious agents within helminth intermediate hosts may prove to be a characteristic of this mechanism of disease transmission. Neither in salmon poisoning nor in blackhead, however, does it seem necessary to provoke or activate the infective agent in order to elicit infection.

The results obtained in the experiments here described may be classified into two groups, depending upon whether or not the experimental swine became clinically ill. In the first group, comprising the original and confirmatory Experiments 1, 2, 3, and 5, the animals developed clinically characteristic swine influenza. The only feature of the disease that differed notably from that seen in intranasally infected swine concerned the distribution of the influenzal pneumonia. In swine receiving their infections experimentally by way of the nose the pneumonia is predominantly localized in the cephalic, cardiac, and azygos lobes with little or no involvement of the diaphragmatic lobes except in cases with a fatal outcome, while in swine receiving their infections through the medium of lungworms the pneumonia was more diffusely distributed throughout the lung and the diaphragmatic lobes especially were involved. In the case of swine 2433 (Experiment 5), killed and autopsied on its first day of illness, only the diaphragmatic lobes were involved and all five of the anterior lobes were completely free of lesions. It is believed, on the basis of the autopsy findings in other swine that were permitted to live through 3 or 4 days of illness and in which the anterior lobes were also involved, that the pneumonia in the diaphragmatic lobes probably represented the initial observable lesion in swine acquiring their virus by way of lungworms. Lungworms are localized almost exclusively in the small bronchi at the bases of the diaphragmatic lobes. From these posterior lobes the infection doubtless spreads, probably by way of the bronchi, eventually involving portions of some or all of the anterior lobes.

Of the second group into which the results fall, were those animals that became immune to swine influenza virus without having exhibited clinical evidence of infection: the animals in Experiments 4 and 6. It is possible that these swine may have undergone attacks of filtrate disease too mild for recognition, but this seems unlikely for two reasons. First, the virus-neutralizing antibody titers were extremely low to represent the result of frank infections even with the swine influenza virus alone; and second, swine 2432 failed to infect by contact swine 2428 in the same pen, a failure that is extremely rare in either swine influenza or filtrate disease. Neither of these findings alone would necessarily eliminate the possibility that the swine in Experiments 4

and 6 had undergone very mild attacks of filtrate disease, but the two considered together make this possibility seem quite unlikely.

The results are more suggestive of those attained in immunizing swine by the subcutaneous or intramuscular administration of virus (20, 21). Such swine develop low titer antibodies and become immune but the virus does not reach the respiratory tract in infective quantities nor spread to other swine by contact from the immunized animals. Such an explanation applied to the results obtained in Experiments 4 and 6 would take into consideration the well known helminthological fact that, among nematode larvae which undergo extensive wanderings in the host's body before reaching their sites of predilection, many are destroyed, or wander into tissues from which they cannot escape. It is believed that the findings with the swine that developed immunity instead of infection can best be explained upon the basis of the activation of virus within lungworm larvae that had been "lost" in non-respiratory tract tissues. There is no reason to suspect, if masked swine influenza virus activated within the respiratory tract results in clinical infection, that that activated outside the respiratory tract should not, like active virus placed there by inoculation, induce an immune response without clinical infection.

It has not as yet been possible to explain the change which takes place in active swine influenza virus when, upon entering the lungworm, it becomes masked. Neither has it been possible to visualize the mechanism whereby the masked virus can again be converted into an infective form upon its return to susceptible swine. That the phenomenon, whatever its mechanism, achieves and insures prolonged survival of the virus is indicated by Experiment 5, in which 17 months elapsed between infection of the swine initially furnishing the virus and the eventual establishment of the virus as an infectious agent in another swine. In like manner, in Experiment 6, 2 years intervened between the swine that originally supplied the virus and the ones that were eventually immunized by it. The swine influenza virus is not to be considered, from its known properties, an unusually resistant agent. It is destroyed in 24 hours by incubation at 37°C. in saline suspension; preserved under favorable conditions in 50 per cent glycerol at refrigerator temperature one cannot depend upon its remaining viable for much longer than 2 months; and in the respiratory tract of an infected swine the virus is not detectable after the 7th day. It is thus apparent that the survival periods recorded for swine influenza virus in the present experiments are very unusual and probably could be duplicated under no set of experimental conditions for storage that might be devised. To the bacteriologically trained person, the possibility that masking of swine influenza virus may be comparable in at least some of its characteristics to spore formation among certain bacteria comes to mind at once. Whatever its nature, the mechanism furnishes a potential means for the preservation of swine influenza virus from one epizootic of swine influenza to the next. In fact

the period of survival of the virus recorded in the present experiments is over twice that which would account for its persistence throughout the usual interepizootic period.

It is believed that the puzzling findings described in the preceding paper (1), in which swine influenza virus infections were elicited in apparently normal swine receiving multiple intramuscular injections of *H. influenzae suis*, can be explained on the basis of the results recorded in the present paper. The swine used in those experiments were observed to have been infested with lungworms. From the way in which those experiments and the ones described in the present paper duplicated one another it appears obvious that the same explanation will hold for both. On this basis masked swine influenza virus present in the lungworms of the swine used is considered to have been accountable for the influenza infections induced.

SUMMARY

1. The swine lungworm can serve as intermediate host in transmitting swine influenza virus to swine. The virus is present in a masked non-infective form in the lungworm, however, and, to induce infection, must be rendered active by the application of a provocative stimulus to the swine it infests. Multiple intramuscular injections of *H. influenzae suis* furnish a means of provoking infection. Swine influenza infections can be provoked in properly prepared swine during the autumn, winter, and spring, but not during the summer. The phenomenon, while not regularly reproducible, occurs in well over half the experiments conducted outside the refractory period of summer. No explanation for the failures is apparent.

2. The virus can persist in its lungworm intermediate host for at least 2 years.

3. Swine infected with swine influenza virus by way of the lungworm intermediate host exhibit a more pronounced pneumonia of the posterior lobes of the lung than do animals infected intranasally with virus. The situation of the worms providing the virus will account for this.

4. Occasional swine infested with lungworms carrying influenza virus fail to become clinically ill after provocation but instead become immune. In these it is believed that lungworms containing the virus are localized outside the respiratory tract at the time of provocation.

5. It is believed that the experiments described furnish an explanation for the findings recorded in the preceding paper, in which swine influenza virus infections were induced in apparently normal swine by multiple injections of *H. influenzae suis*.

6. In a single experiment swine lungworms failed to transmit hog cholera virus.

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INHIBITION OF INCREASE AND ACTIVITY OF TOBACCO-MOSAIC VIRUS UNDER NITROGEN-DEFICIENT CONDITIONS

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INTRODUCTION

Although viruses cause many plant diseases, little is known regarding their metabolism since they apparently multiply only in living tissue. Here it is difficult to differentiate between the metabolism of a host and that of a pathogen. Multiplication of viruses *in vivo* is a well recognized fact and thermal inactivation *in vivo* of a few viruses has been definitely established. No other method for the inactivation of virus *in vivo* has been demonstrated experimentally.

In this study on virus metabolism, a knowledge of the nitrogen metabolism of normal tobacco plants and the discovery that nitrogen supplied to a virus-infected plant had an effect on virus concentration (19) have made it possible to distinguish to some extent between the anabolic and katabolic processes involved. It is well known (14, 24) that in mature leaves of a nitrogen-deficient plant proteins are hydrolyzed and proteolytic decomposition products liberated. These are translocated to the growing point where they supply the meristem with materials necessary for protein synthesis. A study of the effect of withholding nitrogen from a tobacco plant diseased with tobacco mosaic on the total quantity and specific biological activity of virus within the plant has thrown light on the following questions: Is the virus a stable entity under conditions of nitrogen deficiency, or is it subject to normal protein hydrolysis with a subsequent loss in either amount or activity or both? If the virus is hydrolyzed, can the proteolytic products then be used in meeting the normal nitrogen requirements of the plant, or can they be reassembled into fully active virus? A series of experiments were carried out in an effort to determine in what way tobacco-mosaic virus is affected in a nitrogen-deficient plant. Such studies have been made possible by a combination of the techniques of growing plants in nutrient culture, purification of virus by high-speed centrifugation, and estimation of virus activity by a modification of the local lesion method. The results of the studies, a partial summary of which has been published (20), are reported in this paper.

EXPERIMENTAL PROCEDURE

Turkish tobacco plants (*Nicotiana tabacum* L.) were used as host plants in all experiments. Uniform seedlings in the 3- or 4-leaf stage were transplanted into 4-inch porous clay pots filled with washed, white quartz sand. The pots were placed in clay saucers on a bench in a greenhouse, the temperature of which was held between 70° and 80°C. most of the time. Three days after being transplanted, the plants were inoculated with fresh juice from a tobacco plant diseased with tobacco-mosaic virus (*Marmor tabaci* H.).¹ Nutrient treatments were started at time of inoculation. Each plant received 100 ml. of a complete nutrient solution every second day and water between nutrient applications whenever necessary. The nitrogen level of the solution (200 p.p.m. of nitrogen) had been found in previous tests to produce normal growth comparable to that obtained in a rich composted greenhouse soil. The composition of the solution, together with that of a solution deficient only in nitro-

TABLE I
Composition of Nutrient Solutions

| Nutrient solutions | Volume of 0.5 molar stock solutions per liter of nutrient solution | | | | |
|--------------------|--------------------------------------------------------------------|-----------------------------------|-------------------|-------------------------------------------------|-------------------|
| | KH ₂ PO ₄ | Ca(NO ₃) ₂ | MgSO ₄ | (NH ₄) ₂ SO ₄ | CaCl ₂ |
| | ml. | ml. | ml. | ml. | ml. |
| Complete | 12.9 | 11.0 | 4.0 | 3.3 | 0.0 |
| Minus nitrogen | 12.9 | 0.0 | 4.0 | 0.0 | 11.0 |

gen, reference to which will be made later, is given in table I. In addition to the salts listed in the table, each solution was supplemented with $\frac{1}{2}$ p.p.m. of both boron and manganese as H₃BO₃ and MnSO₄, respectively.

At intervals, representative plants were cut, weighed, and then placed in covered pans in a cold room held at -14°C. Twenty-four hours later the frozen plants were minced in a food chopper. The pulp was mixed with 3 per cent. by weight of K₂HPO₄ and allowed to thaw at room temperature. The cold juice was expressed from the pulp through cheese cloth by means of a small screw press and cleared of all extraneous insoluble materials by low-speed centrifugation. Aliquots of this juice were then assayed for virus activity, total protein, and total virus protein. The assay of plant proteins included all soluble protein fractions. No account was taken of the insoluble fractions.

Virus activity of juice from diseased plants was assayed by means of the local lesion method (4), using bean (*Phaseolus vulgaris* L. var. Early Golden Cluster) as the test plant (16). Although the use of this method does not give

¹ Latin names of viruses used in this paper were taken from the Handbook of Phytopathogenic Viruses (5).

a measure of the absolute amount of active virus present, certain modifications have recently been made by means of which it is possible to estimate, usually within 10 to 20 per cent., the quantitative difference in virus activity between two or more samples. Data on this modified method and experiments pertaining to its accuracy will be published later (22).

Total soluble protein content of juice was determined by precipitation of the protein by hot trichloroacetic acid. One ml. of juice was treated with 1 ml. of hot 5 per cent. trichloroacetic acid and immediately cooled. Following centrifugation, the denatured protein was dissolved in 1 ml. of 0.2 N NaOH, reprecipitated with 1 ml. of 10 per cent. trichloroacetic acid and again centrifuged. The protein precipitate was transferred to a micro-Kjeldahl flask with 1 ml. of 0.2 N NaOH and analyzed for Kjeldahl nitrogen by the modified method (11, 15) of Folin and Farmer (3), as follows: To the protein solution was added 2 ml. of concentrated H_2SO_4 , 1 gm. of K_2SO_4 , 2 drops of SeOCl_2 , and a few alundum chips. The mixture was digested for at least 5 minutes after the contents had become clear or straw-colored. After the addition of 30 ml. of water and 6 ml. of 50 per cent. NaOH, nitrogen as ammonia was distilled over into 0.02 N HCl. All titrations were made with 0.02 N NaOH, using methyl red as an indicator. This determination included both soluble plant protein and virus protein.

The virus protein was isolated by means of an air-driven ultracentrifuge as previously described (21). Stainless steel centrifuge tubes were used in place of thin-walled celluloid tubes. The content of sedimented virus, as measured by the virus-protein content, was then assayed by digestion of the virus protein with H_2SO_4 , K_2SO_4 , and SeOCl_2 , as outlined above.

This experiment was carried out during September and October. Similar experiments conducted during November and December and again during early summer gave comparable results.

RESULTS

A study was first made of the effect of nitrogen starvation on young plants showing severe systemic symptoms of tobacco mosaic. Starting at time of inoculation, all plants were fed the complete nutrient solution. Ten days after inoculation the plants were divided into two groups. One group continued to receive the complete nutrient solution every second day. The other group, after a thorough flushing of the sand to remove as much nitrogen as possible, was fed the minus-nitrogen solution every second day. At 4-day intervals, representative plants from each group were harvested and analyzed for total protein, virus protein, and virus activity.

In table II are presented data pertaining to the growth of diseased plants that received the two nutrient treatments. Four days after transfer, the nitrogen-fed plants weighed about 15 per cent. more than those deprived of

nitrogen. With each succeeding harvest thereafter, the difference in size of plants between the two groups became progressively greater, until by the 24th day the nitrogen-fed plants were nearly 5 times the size of those deficient in nitrogen. During this 24-day period, the green weight of nitrogen-fed plants increased about 18-fold, whereas that of nitrogen-deficient plants increased less than 4 times. A portion of this increase in nitrogen-deficient plants was probably due to the reserve of unassimilated nitrogen present in these plants at the time they were transferred to the minus-nitrogen treatment.

Table II also shows the virus activity of juice from the nitrogen-deficient plants relative to that of juice from nitrogen-fed plants of the same age at each of the 6 harvests. In each case the activity of the nitrogen-fed plants

TABLE II

Data on Growth of Diseased Tobacco Plants and on Relative Virus Activity of Their Juices, When Harvested at Intervals after Transfer to the Minus-Nitrogen Treatment

| Time of harvest: days after transfer to minus-N treat- ment* | Number of plants harvested | | Average per plant | | | | Virus activity† of N-deficient plants per | |
|--------------------------------------------------------------------------|-------------------------------|------------------|-------------------|------------------|------------------------------|------------------|-------------------------------------------------|-------|
| | | | Green weight | | Volume of juice expressed | | | |
| | + N treatment | - N treatment | N-fed plants | N-def. plants | N-fed plants | N-def. plants | Ml. of juice | Plant |
| <i>days</i> | | | <i>gm.</i> | <i>gm.</i> | <i>ml.</i> | <i>ml.</i> | % | % |
| 0 | 20 | | 2.9 | | 2.0 | | | |
| 4 | 12 | 12 | 5.4 | 4.7 | 3.0 | 2.8 | 100 | 93 |
| 8 | 10 | 15 | 9.5 | 5.2 | 5.5 | 3.7 | 64 | 43 |
| 12 | 6 | 10 | 16.2 | 7.5 | 9.0 | 4.7 | 54 | 28 |
| 16 | 5 | 10 | 24.8 | 7.6 | 17.8 | 4.6 | 87 | 22 |
| 20 | 5 | 10 | 38.2 | 10.3 | 26.8 | 6.4 | 64 | 15 |
| 24 | 5 | 7 | 52.8 | 11.1 | 34.0 | 7.0 | 32 | 7 |

* Minus-nitrogen treatment started 10 days after inoculation.

† Virus activity relative to that of N-fed plants harvested at same time.

was assumed equal to 100 per cent. The virus activity per ml. of juice from plants on the minus-nitrogen treatment for 4 days was apparently equal to that of nitrogen-fed plants. After 24 days on the minus-nitrogen treatment, however, the juice from these plants was only $\frac{1}{5}$ as active on a unit volume basis as was that from plants receiving an adequate nitrogen supply. The last column in table II shows the calculated relative virus activity of the nitrogen-deficient plants at each of the harvests. These calculations are based on the volume of juice expressed per plant and the relative virus activity per ml. As shown by the data, the difference in virus activity between the two nitrogen treatments became greater and greater, until by the 24th day the nitrogen-fed plants had nearly 15 times as much virus as did nitrogen-deficient plants of the same age. Possible explanations for this large difference will be discussed in a later section.

Data pertaining to the total-protein content and the virus-protein content of aliquots of juice from the two sets of plants at each harvest are recorded in table III. In plants receiving nitrogen, the mg. of total protein per ml. of juice increased at first and then decreased slightly. The low protein contents recorded on the 16th and 24th day were probably due in part to the fact that each of these harvest days was preceded by one or more dark, cloudy days. The decrease in carbohydrate synthesis is accompanied by an accelerated hydrolysis of stored proteins. In nitrogen-deficient plants a steady decrease took place in the mg. of total protein per ml. This decrease was apparently brought about by a dilution of synthesized protein with subsequent growth and

TABLE III

Content of Total Protein and Content and Relative Activity of Twice-Ultracentrifuged Virus Protein in Juice from Diseased Plants Harvested at Intervals after Transfer to the Minus-Nitrogen Treatment

| Time of harvest days after transfer to minus- N treatment* | Total protein (mg. per ml.) | | Virus protein (mg. per ml.) | | Activity† of virus protein from N-def. plants per unit weight of protein | Activity† of N-def. plants as calculated from virus protein data |
|---------------------------------------------------------------------|--------------------------------|-----------------|--------------------------------|-----------------|--------------------------------------------------------------------------------------|------------------------------------------------------------------------------|
| | N-fed plants | N-def plants | N-fed plants | N-def plants | | |
| <i>days</i> | | | | | % | % |
| 0 | 11.1 | | 2.7 | | | |
| 4 | 9.5 | 9.3 | 3.0 | 3.5 | 100 | 109 |
| 8 | 11.7 | 8.0 | 4.4 | 4.0 | 71 | 43 |
| 12 | 12.1 | 6.1 | 5.2 | 3.4 | 69 | 24 |
| 16 | 9.2 | 5.7 | 3.7 | 3.1 | 84 | 18 |
| 20 | 11.1 | 5.1 | 4.5 | 2.8 | 88 | 13 |
| 24 | 9.7 | 3.7 | 4.4 | 2.4 | 58 | 7 |

* Minus-nitrogen treatment started 10 days after inoculation.

† Virus activity relative to that of N-fed plants harvested at same time.

not to an actual loss of protein. Evidence substantiating this conclusion will be presented later. The virus data show that in nitrogen-fed plants the mg. of virus protein per ml. of juice increased up to the 12th day and then decreased slightly. This slight decrease may be due to an inadequate supply of nitrogen to insure a continuation of the same growth rate in such large plants. The mg. of virus protein per ml. of juice from nitrogen-deficient plants increased slightly at first and then decreased. As in the case of the total protein in these plants, the apparent decrease is only a dilution effect. The composite protein data show that at the last harvest 40 per cent. of the total protein in juice of the nitrogen-fed plants was virus protein, whereas in juice from plants starved for nitrogen, from 60 to 65 per cent. of the total protein was virus protein. This yield of virus is lower than that obtained by Stanley (23), who reported a yield of crystalline virus equal to 80 per cent. of the total protein in the juice. No explanation for this discrepancy has been found.

The virus data discussed so far in table III indicate the virus-protein content in the juices from the two sets of plants but give no information regarding the activity of this virus protein. The fact that virus protein is not broken down does not necessarily imply that the virus is still active, for virus inactivation may not be synonymous with virus disintegration. As Lauffer and Price have recently suggested (10), virus inactivation by heat is not identical with thermal denaturation of the virus but may be one of a series of reactions that eventually lead to denaturation. In their heat studies, carried out *in vitro*, virus inactivation proceeded at a faster rate than thermal denaturation.

The 6th column of table III records the biological activity on a unit weight basis of virus protein from the nitrogen-deficient plants relative to that of virus protein from plants receiving the complete nutrient solution. Four days after transfer, the activities per unit weight of virus from the two sets of plants were practically identical. Four days later, virus from the nitrogen-deficient plants was only 71 per cent. as active as that from nitrogen-fed plants. By the 24th day virus from nitrogen-deficient plants was only 58 per cent. as active on a unit weight basis. It might appear as though this value were unusually low in view of the values on the 16th and 20th day, but a later experiment indicated that these two values were not representative and that the 58 per cent. was approximately correct. As will be brought out later, this loss of nearly half the activity was not accompanied by any detectable denaturation or hydrolysis of virus even when nitrogen was withheld from the plant for 24 days.

In the last column of table III is shown the relative virus activity of the entire plant at each harvest, as calculated from the content of virus protein in the juice per plant and the relative activity per unit weight of this virus protein. These calculations show that after the first few days following the transfer from the complete nutrient to the minus-nitrogen treatment, the difference between the virus activities of the two treatments increased very rapidly. By the time the plants had been on the minus-nitrogen treatment for 24 days, juice from these plants was only 7 per cent. as active as that from the nitrogen-fed plants. It is worth noting that these values are essentially the same as those presented in the last column of table II. Although the same entity was measured in both cases, it is interesting that the two methods, one based on measurements with crude juice and the other based on measurements with purified virus, check each other so closely. In this connection it should be pointed out that only a small fraction of the difference between the virus activities of the two groups of plants was due to loss of activity of the virus on the part of the nitrogen-deficient plants. Most of the difference was due to an increase of virus in the nitrogen-fed plants.

In the experiments so far reported, a number of diseased plants was trans-

ferred to the minus-nitrogen treatment 10 days after inoculation. In a second series of experiments, carried out in conjunction with those discussed above, tests were made with plants transferred to the minus-nitrogen treatment 18 days after inoculation. These plants were more than 3 times the size of those changed at the earlier date and were growing much more rapidly at the time of transfer. Representative nitrogen-fed and nitrogen-deficient plants were harvested every 4 days and assayed as in the first series of experiments. The analytical data obtained are recorded in table IV.

It is apparent from these data that the results obtained with the larger plants are very similar to those recorded with the smaller plants. The nitro-

TABLE IV

Virus Activity and Protein Contents of Juice from Nitrogen-Fed Plants and from Nitrogen-Deficient Plants at Intervals after Initiation of Minus-Nitrogen Treatment, Which Was Started 18 Days after Inoculation

| Time of harvest days after transfer to minus-N treatment | Number of plants harvested | | Volume of juice expressed per plant | | Total protein per ml of juice | | Virus protein per ml of juice | | Activity* of N-deficient plants per | | Activity* of N-def plants as assayed with | |
|----------------------------------------------------------|----------------------------|--------------|-------------------------------------|--------------|-------------------------------|--------------|-------------------------------|--------------|-------------------------------------|--------------------|-------------------------------------------|---------------|
| | N-fed plants | N-def plants | N-fed plants | N-def plants | N fed plants | N-def plants | N-fed plants | N-def plants | ml of juice | Unit wt of protein | Crude juice | Virus protein |
| days | | | ml | ml | mg | mg | mg | mg | % | % | % | % |
| 0 | 10 | | 5 5 | | 11 7 | | 4 4 | | | | | |
| 4 | 6 | 6 | 9 0 | 9 5 | 12 1 | 9 6 | 5 2 | 4 4 | 76 | 74 | 80 | 66 |
| 8 | 5 | 5 | 17 8 | 13 4 | 9 2 | 6 9 | 3.7 | 3 5 | 67 | 79 | 50 | 56 |
| 12 | 5 | 5 | 26 8 | 14 4 | 11 1 | 6 1 | 4 5 | 3 2 | 67 | 86 | 36 | 33 |
| 16 | 5 | 7 | 34 0 | 15 4 | 9 7 | 6 2 | 4 4 | 3 6 | 54 | 83 | 24 | 31 |
| 20 | 5 | 6 | 42 2 | 14 3 | 10 4 | 5 6 | 4 2 | 3 1 | 55 | 68 | 19 | 17 |

* Virus activity relative to that of nitrogen-fed plants harvested at the same time.

gen-fed plants increased nearly 8 times in size during the 20-day period; the nitrogen-deficient plants little more than doubled in size. Size was measured by the volume of juice expressed, since this was roughly proportional to the green weight of the plants. The mg. of total protein and of virus protein per ml. of juice remained practically constant in the nitrogen-fed plants but decreased markedly in juice from the nitrogen-deficient plants. At the end of the experiment, 40 per cent. of the protein present in juice from nitrogen-fed plants was present as virus protein, whereas in the juice from nitrogen-deficient plants 55 per cent. of the total protein was accounted for as virus protein. The virus protein in nitrogen-deficient plants was only 70 per cent. as active after the 20-day period on the minus-nitrogen treatment, but no decrease in total mass of virus protein could be detected.

The virus-protein data so far considered have been based entirely on the

mg. per ml. of expressed juice and, as such, give only a fragmentary picture of what took place within the plants. In order to fill in the picture, it is necessary to consider total yield of virus protein and of soluble protein per plant as extracted in the plant juice. The yields of virus protein and of total soluble protein in juice from plants on the two treatments at each of the several harvests are represented graphically in figure 1.

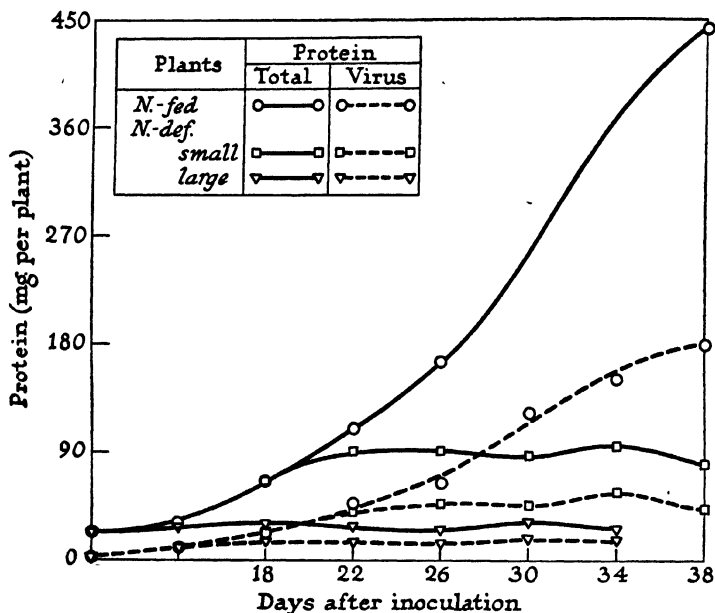


FIG. 1. Total-protein and virus-protein contents in juice from nitrogen-fed and nitrogen-deficient plants at intervals following inoculation with tobacco-mosaic virus.

As shown by the upper line, the protein content of nitrogen-fed plants increased slowly at first while the plants were small, but as the plants became larger, the protein content increased more markedly. This indicated a rapid synthesis of proteins in these plants. A similar trend was shown by the virus-protein content in these plants as measured at 4-day intervals. The increase appears to be more gradual, whereas in reality the percentage increase was actually much greater. In the 24-day period during the test, the total protein increased about 15 times—from 22 mg. to 330 mg., whereas the virus protein increased 30 times—from 5 mg. to 150 mg.

The graphic representation of data pertaining to the protein content of plants transferred to the minus-nitrogen treatment 10 days after inoculation

displayed an entirely different trend. After a small initial rise during the first 8 days on the minus-nitrogen treatment, both the content of soluble protein and that of virus protein remained practically constant during the remainder of the experimental period of 16 days. These data show that the decrease in mg. of protein per ml. of juice, as recorded in table III, was due to a dilution of the protein already synthesized, with subsequent growth, rather than to a loss of this protein, since the protein content of the plant apparently remained constant. Graphs with a similar trend were found when the contents of total protein and virus protein in juice from plants held on the plus-nitrogen treatment for 18 days before being changed to the minus-nitrogen treatment were plotted.

From the 4 graphs in figure 1, obtained by plotting data from nitrogen-deficient plants, it is possible to draw several tentative conclusions regarding the nitrogen metabolism of a virus-infected plant. In the first place, even a plant suffering from severe nitrogen deficiency is apparently unable to utilize, in the synthesis of its normal proteins, any of the nitrogen in the virus, since its non-virus protein content does not increase and its virus-protein content does not decrease. Some change, however, does take place in the virus in such a plant to render it partially inactive without bringing about any detectable decrease in yield of virus protein per plant. On the other hand, the virus is apparently unable to utilize any measurable part of the soluble proteins of the plant, for these constituents do not decrease. It is apparent, therefore, that the plant and virus may have competed against each other for the nitrogen absorbed by the plant. Such an hypothesis would explain why a mosaic-diseased plant shows symptoms of nitrogen deficiency at an earlier date than does a healthy plant grown under the same condition. Part of what little nitrogen is available in such plants would be utilized by the virus, rendering the deficiency condition even more severe.

DISCUSSION

Several workers (1, 13, 17, 23) have suggested either directly or indirectly that in a mosaic-diseased plant normal proteins may be destroyed or utilized in the formation of virus. This hypothesis was not substantiated by observations in the present study in which it was found that the content of normal proteins in a diseased plant receiving no additional nitrogen did not decrease but remained more or less constant throughout the experimental test period. Although these observations were based on the soluble protein content of the expressed juice, the evidence showed that in the absence of an external supply of nitrogen no further virus multiplication could be detected. This would indicate that no conversion of soluble or insoluble normal proteins into virus took place under these conditions. Virus may multiply, however, at the expense of normal protein by utilizing in its metabolism some of the nitrogen

absorbed by the plant before the plant can assimilate such nitrogen in the synthesis of normal proteins.

It has been reported (13, 17) that virus protein increases and then decreases with increasing maturity of the diseased plant. The evidence, which has been interpreted to show a destruction of virus, was based on the virus content per gm. of tissue. Similar results have been obtained (6, 18, 19) using crude juice. Data obtained in the present paper indicated the same trend, but when these data were calculated on a plant basis it was obvious that, in the absence of further multiplication, the virus content showed no decrease but remained constant. The above-mentioned papers do not contain sufficient growth data to permit such a calculation. The reduction per unit weight or per unit volume is apparently due to a dilution of the virus with subsequent growth of the plant and not to an actual loss or destruction of virus. The data in this paper show that the activity of the virus may decrease under certain conditions. Such an inactivation has not been demonstrated previously.

It is difficult to reconcile the results of the writer with those of Rischkov and Smirnova (17), who reported that, in tomato plants deficient in nitrogen, virus not only continued to accumulate but even reached a concentration equal to that present in plants supplied normal amounts of nitrogen. The data presented here (table III and fig. 1) show a much higher virus content in nitrogen-fed plants than in nitrogen-deficient plants.

The partial inactivation of tobacco-mosaic virus in a nitrogen-deficient plant is interesting because of the relative stability of this virus *in vitro*. Two diseases of sugar cane, sereh disease of Java (7), and chlorotic streak (12), have been cured by heat treatment. The former is believed to be caused by a virus, but some doubt exists as to whether or not chlorotic streak is a virus disease (2). Recent experiments with such well recognized viruses as those of aster yellows (9), peach yellows, little peach, red suture, and peach rosette (8) have definitely established the fact that these viruses can be inactivated *in vivo* by heat. Ross (18) has recently reported a marked decrease with age of plant in virus activity of crude juice from tobacco plants diseased with alfalfa-mosaic virus (*Marmor medicaginis* H.). Since this decrease was too rapid to be due to a diluting effect of growth, he concluded that the virus was partially inactivated. No data were reported concerning the content and biological activity of sedimentable virus protein in these plants.

At present writing it is impossible to advance any definite explanation for the inactivation of virus *in vivo*. Ross (18) believes that the virus of alfalfa mosaic may have been inactivated by heat, since this virus has a low thermal inactivation point *in vitro* and since the virus never reaches a high concentration in tobacco plants grown during the summer months. Such an explanation would not hold in the case of tobacco-mosaic virus, as this virus is one of the most stable plant viruses so far studied, with a thermal inactivation point of about

90°C. If the virus is a living entity, it is possible that a portion may become avirulent or die out but not be disintegrated, thereby accounting for the loss in activity without a decrease in mass. If the virus is a protein molecule, its biological activity may be dependent on the presence of one or more active groups, and the loss or alteration of such a grouping may occur without a breaking apart of the macromolecule. It is apparent, however, that as far as the plant in a nitrogen-deficient condition is concerned, the virus protein acts as a foreign protein since it is not subject to normal proteolytic hydrolysis by the plant systems. Further work is necessary to elucidate the manner in which virus inactivation takes place in a nitrogen-deficient plant.

SUMMARY

Experiments were carried out to determine in what way tobacco-mosaic virus is affected in a plant deficient in nitrogen. Turkish tobacco seedlings, grown in sand cultures and supplied a complete nutrient solution (nitrogen level = 200 p.p.m.), were inoculated with tobacco-mosaic virus. Ten days after inoculation the plants were divided into two groups; one group (nitrogen-fed plants) continued to receive the complete solution and the second group (nitrogen-deficient plants) received a nutrient solution complete in everything but nitrogen. Representative plants from each group were harvested at 4-day intervals and the expressed juice assayed for relative virus activity, total protein, and virus protein.

In nitrogen-deficient plants the virus-protein content as well as the content of soluble plant protein remained practically constant, whereas in nitrogen-fed plants each increased more than 5 times during a 16-day period. Although no decrease in the yield of virus protein in the nitrogen-deficient plants could be detected, its biological activity, however, decreased more than 40 per cent. It is not known how this inactivation was brought about.

As far as the nitrogen-deficient plant is concerned, the virus protein acts as a foreign protein, for the virus was apparently not affected by the normal proteolytic processes of the plant. Even a plant suffering from a severe nitrogen deficiency was unable to use, in the synthesis of its normal proteins, any nitrogen previously utilized by the virus. On the other hand, the virus was unable to utilize any nitrogen tied up in the proteins normally present in a nitrogen-deficient plant, for in the absence of an external supply of nitrogen, no further virus multiplication could be detected.

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THE DETERMINATION OF SOME AMINO ACIDS IN TOBACCO MOSAIC VIRUS PROTEIN

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Several plant viruses have been isolated in the form of characteristic nucleoproteins of high molecular weight (1-5). The properties of many of these have been determined and compared, but with few exceptions (6, 7) analytical data have been limited to elementary analyses and to determinations of the amount and kind of nucleic acid present. Comparatively little is known of the protein components which comprise the greater part of the virus molecules. A better understanding of the chemical structure that imparts to viruses the ability to reproduce and to mutate, properties also characteristic of accepted living organisms, would obtain if the components of which the different viruses are built were known. It is important to know the number and kind of reactive groups present in different types of virus molecules, the manner in which viruses resemble one another in composition and how they differ, and the respects in which they differ from other proteins. Since many viruses mutate, giving rise to strains which are closely related proteins, analytical data on such proteins might relate changes in biological, chemical, serological, and physical properties to changes in composition. It is possible that specificity of host range (8) may be explained by the ability or inability of a particular species of plant to provide certain amino acids required by a given virus. In this paper are reported some of the analyses for amino acids that have been made on tobacco mosaic virus protein. While a complete analysis of the protein component has not been obtained, a fair proportion of the molecule has been accounted for and some information concerning the nature of the remainder has been obtained. It is recognized that some of the

values presented may differ somewhat from values that may be obtained in the future by improved methods (9). However, the data make possible a comparison with other proteins at the present time, and it is hoped that they will constitute a basis for a comparative examination of other strains of this virus and of other viruses. A preliminary report of part of this work has previously (10) been given.

EXPERIMENTAL

Preparation of Material—The preparations previously described (6) were used in most instances, although during a part of the work additional samples were prepared by the same methods. All preparations were dried to a moisture content of about 8 per cent by freezing and drying *in vacuo*. All determinations were based on samples dried to constant weight at 110°. Unless otherwise specified, a Goudsmit-Summerson photoelectric colorimeter manufactured by the Klett Manufacturing Company, Inc., was used.

Tyrosine and Tryptophane—The Folin-Marenzi (11) method was found unsatisfactory, for cloudy solutions were obtained. Lugg's (12) modification of the method gave reproducible results. Virus samples weighing 200 to 300 mg. were hydrolyzed as described by Bailey (13) and aliquots containing 1 to 2 mg. of tyrosine were taken for comparison. A few determinations were made by means of Bernhart's (14) micromethod for tyrosine and Shaw and McFarlane's (15) glyoxylic acid method for tryptophane. In the last named method, 50 mg. of virus were dissolved in 1 cc. of 20 per cent sodium hydroxide. This solution was made to 25 cc. and an aliquot portion removed for color development and comparison with a tryptophane standard. In the Bernhart and glyoxylic acid methods, a Klett-Summerson photoelectric colorimeter with a green filter was used. The results are presented in Table I. A tyrosine content of 3.9 per cent was indicated by both methods. In the case of tryptophane, a given method gave reproducible results, but the Folin-Marenzi-Lugg method gave lower results than did the other procedure. The discrepancy may be due to losses during hydrolysis, to the fact that the color fades quite rapidly, or to the presence of an interfering substance in this particular protein. Shaw and McFarlane consider that tryptophane combined in a protein gives the same amount of color as

does the free acid, but it is possible that in some combinations the color formation may be enhanced. However, for the present, the higher value of 4.5 per cent will be regarded as more nearly correct.

Arginine—Arginine was determined by Weber's (16) modification of the Sakaguchi (17) reaction. The protein was hydrolyzed by boiling for 20 hours with 20 per cent HCl; humin was removed by filtration and then well washed. Aliquot portions of the combined filtrate and washings were diluted and aliquots containing

TABLE I
Tyrosine and Tryptophane Content of Tobacco Mosaic Virus

| Amino acid | Method | Amount in virus* | | |
|-------------|------------------------|------------------|-----------|-----------|
| | | Sample 1† | Sample 2‡ | Sample 3‡ |
| Tyrosine | Folin-Marenzi-Lugg | per cent | per cent | per cent |
| | | 3.9 | 3.9 | |
| | | 3.9 | 3.9 | |
| | Bernhart's micromethod | 3.7 | 3.8 | |
| | | 3.8§ | 3.9§ | 3.9§ |
| Tryptophane | Folin-Marenzi-Lugg | 3.8 | 3.8 | 3.9 |
| | | 1.9 | 2.0 | |
| | | 2.1 | 2.0 | |
| | Glyoxylic acid method | 2.0 | 2.1 | |
| | | 4.6§ | 4.5§ | 4.4§ |
| | | 4.5 | 4.5 | 4.5 |

* The figures in these columns are the averages of two or more analyses of a hydrolysate or solution.

† Ultracentrifugally isolated.

‡ Chemically isolated.

§ Dr. C. A. Knight of this laboratory has recently obtained similar results with these and with other samples of tobacco mosaic virus.

0.004 to 0.01 mg. of arginine were taken for the determinations. The results, which are presented in Table II, indicate an arginine content of 9.0 per cent. Arginine was precipitated from other hydrolysates by the method of Van Slyke (18) and the precipitate dissolved in dilute alkali. Aliquot portions of this were taken for analysis. The remainder was freed of phosphotungstic acid by the method of Van Slyke and the resulting solution also analyzed for arginine. The results were only slightly lower than those obtained with the diluted hydrolysates. The value here reported

has also been confirmed by the isolation of arginine by means of flavianic acid. The details will appear in a later paper.

Histidine—The fractions described above that were used for arginine determinations were also used for tests for histidine. Jorpes' (19) modification of the Pauly (20) reaction and the Kapeller-Adler (21) test as modified by Woolley and Peterson (22) both gave negative results with one exception. In that one case, sufficient tyrosine was found to be present to give the test. An amount

TABLE II
Arginine and Histidine Content of Tobacco Mosaic Virus

| Amino acid | Method | Fraction used for determination | Amount found ^a |
|------------|-------------------------|--------------------------------------|---------------------------|
| Arginine | Sakaguchi-Weber | Acid hydrolysate diluted | per cent |
| | | | 9.0 |
| | | | 9.1 |
| | | | 9.1† |
| | | | 9.1† |
| Histidine | Pauly-Jorpes | Phosphotungstic acid ppt. | 8.9 |
| | | Decomposed phosphotungstic acid ppt. | 8.9 |
| | | Phosphotungstic acid ppt. | 0.0 |
| | | Decomposed phosphotungstic acid ppt. | 0.04‡ |
| | Modified Kapeller-Adler | “ “ | 0.0 |
| | | “ “ | Trace‡ |

^a Each figure in this column is the average of two or more determinations.

† An ultracentrifugally isolated sample was used for analysis. In all other cases, a chemically isolated sample was used.

‡ Probably due to the presence of a trace of tyrosine.

of histidine equivalent to less than 0.1 per cent of the protein could be recovered when added to the fractions. Hence, it appears that histidine is not present in the tobacco mosaic virus molecule.

Phenylalanine—Phenylalanine was determined by Block's (23) modification of the Kapeller-Adler (24) reaction. The test was applied directly to acid hydrolysates and also to the monoamino acid fraction obtained by the Van Slyke (18) procedure. The two methods gave somewhat different values, as may be seen in Table III. The values are probably only approximations, for the

intensity of color development is influenced by other amino acids. Block added glycine to standards, a procedure which caused enhanced color development. In the present work, the addition of leucine resulted in an inhibition of color formation. The values reported were obtained with controls to which no amino acid other than phenylalanine was added.

Serine—Samples of the virus protein were hydrolyzed by boiling with 20 per cent HCl for 20 hours. The excess HCl was removed

TABLE III
Determination of Some Amino Acids in Tobacco Mosaic Virus

| Amino acid | Method | Fraction used for determination | Amount found* |
|---------------|-------------------------------|--------------------------------------------|-----------------|
| | | | <i>per cent</i> |
| Phenylalanine | Block | H ₂ SO ₄ hydrolysate | 6.6 |
| | | | 6.7† |
| | | Van Slyke monoamino acid fraction | 5.6 |
| Serine | Rapoport | " " | 6.4 |
| | | | 6.5† |
| | | | 5.7 |
| Glycine | <i>o</i> -Phthalaldehyde | Acid hydrolysate | None |
| | Potassium trioxalatochromiate | " " | " |
| Threonine | Block-Bolling | " " | 5.2† |
| | | | 6.2 |
| | | | 4.6 |
| Proline | Bergmann's solubility method | " " | 4.67 |
| | | | 4.66† |

* Each figure in this column represents the average of two or more analyses of a hydrolysate.

† Ultracentrifugally isolated sample. All others were chemically isolated.

from the hydrolysates by repeated evaporation under reduced pressure. The basic amino acids were removed by precipitation with phosphotungstic acid (18) and the dicarboxylic acids by the method of Jones and Moeller (25). The solutions were freed of barium, then analyzed for serine by the method of Rapoport (26) in which serine is deaminated, and then condensed with naphthoresorcinol. The results indicate that the protein is unusually rich in serine; if the method is assumed to be accurate and glycine

is in fact not present, the presence of about 6.4 per cent serine is suggested.

Glycine—The *o*-phthalaldehyde reaction of Zimmermann (27) was used for the estimation of glycine. The protein was hydrolyzed and the test applied as described by Patton (28). No chloroform-soluble color developed, although the test was repeated several times. When an amount of glycine equivalent to less than 0.5 per cent of the protein was added to the hydrolysate, a chloroform-soluble color resulted, but it did not exactly match the standard. The results are indicative, but not proof, of the absence of glycine in the virus protein.

Potassium trioxalatochromiate was also used in several attempts to precipitate glycine by the method described by Bergmann and Fox (29). In a typical experiment, 9.8 gm. of chemically isolated protein were hydrolyzed by boiling 20 hours with 20 per cent HCl. The hydrolysate was freed of excess HCl by repeated evaporations under reduced pressure, then adjusted to pH 2 with silver oxide. The silver chloride was washed with hot water and the combined washings and filtrate concentrated to 26 cc. To this, 6.2 cc. of 10 per cent HCl, 2 gm. of potassium trioxalatochromiate, and 46 cc. of absolute alcohol were added. The mixture was shaken for 8 hours, then placed in a cold room overnight. The precipitate, when collected on a filter, washed with a cold 3:1 mixture of absolute alcohol and 0.5 N HCl, and dried in air, weighed 0.7397 gm. However, it contained but 0.14 per cent amino nitrogen, corresponding to less than 0.1 per cent glycine in the protein. Under comparable conditions, 78 per cent recovery was obtained with 100 mg. of glycine. The experiment was repeated several times, with smaller amounts of the reagent as well as different concentrations of hydrolysate, and similar results were obtained. The amount of amino nitrogen in the precipitate was always quite low; hence it seems probable that it was due to small quantities of other amino acids adsorbed on the excess reagent that precipitated. When a quantity of glycine equivalent to about 1 per cent of the protein was added to a hydrolysate, most of it could be recovered. The data indicate that glycine is not present in the virus protein.

Threonine—Threonine was determined by the method of Block and Bolling (30). Considerable variation in results was encountered, as may be seen in Table III. The values obtained are

probably a rough estimate of the amount of threonine in this protein, but it is possible that acetaldehyde may have arisen from other sources.

Hydroxyproline and Proline—The reaction for hydroxyproline was applied according to the directions of McFarlane and Guest (31), except that hydrolysis was accomplished by boiling for 20 hours with 20 per cent HCl. Negative results were obtained in all cases with both chemically and ultracentrifugally isolated samples. However, when hydroxyproline was added to the hydrolysates, the test was still negative. The procedure was varied in many ways and a partial separation of hydroxyproline from other amino acids was made, but the results were invariably negative. Hence, this test is not applicable to tobacco mosaic virus hydrolysates. Proline was determined by the method of Guest (32), with hydrolysates prepared for the hydroxyproline tests. The results indicated about 8 per cent proline. However, this value is higher than that obtained by other means, and since hydroxyproline also gives a positive test, it appears probable that the latter amino acid is present in the virus molecule.

Bergmann and Stein's (33) solubility method was also utilized in the determination of proline. In one experiment, 11.48 gm. of the chemically isolated virus were hydrolyzed by boiling 18 hours with 20 per cent HCl. The hydrolysate was freed of excess HCl and decolorized as described by Bergmann and Stein. It was then concentrated under reduced pressure, 1 cc. of concentrated HCl was added, and the solution adjusted to 100 cc. 20 cc. portions of methyl alcohol containing 0.3584, 0.4578, and 0.5824 gm., respectively, of ammonium rhodanilate¹ were added to three separate samples each of 32 cc. of the hydrolysate. The flasks were stoppered, allowed to stand 16 hours at 0°, then shaken for 4 hours at 4°, and again allowed to stand for 24 hours at 0°. The precipitates were collected by filtration at 0° on sintered glass funnels, washed with 4 cc. of ice-cold water, and then dried in air and finally to constant weight over CaCl₂ and NaOH. When calculated by the method of Bergmann and Stein, values of 4.74, 4.64, and 4.64 per cent, average 4.67 per cent, were obtained. The results from a similar experiment averaged 4.66 per cent.

¹ The reagent was prepared as described by Bergmann (34) and repurified before use by the method of Bergmann and Stein (33).

DISCUSSION

The significance of amino acid analyses is dependent, of course, upon the purity of the protein. The fact that no differences were detected between the chemically isolated samples and those isolated by means of the ultracentrifuge indicates that the samples were essentially pure. If significant quantities of impurities were present in one type of sample, it seems probable that the same kind and quantity would not be contained in a sample isolated by an entirely different method. Furthermore, considerable evidence has accumulated indicating that ultracentrifugally isolated virus protein approaches a high degree of homogeneity and contains no significant quantities of impurities.

Since the tobacco mosaic virus molecule has a molecular weight of about 43 million (35), there is little to be gained by calculating the frequencies of the amino acid distribution by the method of Bergmann and Niemann (36). The suggestion has been made by Astbury (37) that the virus may be made up of uniform subunits. If this is true, the smallest possible uniform unit, based on a cysteine content of 0.68 per cent (6), would correspond to a molecular weight of about 17,800, a unit considerably larger than that suggested by Astbury. Of the several determinations reported in this paper, those for arginine, tryptophane (glyoxylic acid method), and for tyrosine gave results in fair agreement with Bergmann and Niemann's hypothesis based on the above subunit.

SUMMARY

Tobacco mosaic virus has been found to contain 3.9 per cent tyrosine, 4.5 per cent tryptophane, 4.7 per cent proline, 9.0 per cent arginine, 6.7 per cent phenylalanine, 6.4 per cent serine, and about 5.3 per cent threonine. Glycine and histidine appear to be absent. No differences were noted between chemically isolated samples and those isolated by means of the ultracentrifuge.

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PURIFICATION AND PROPERTIES OF ALFALFA-MOSAIC VIRUS PROTEIN

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Since the isolation of tobacco-mosaic virus protein by Stanley in 1935 (16), several other virus nucleoproteins have been obtained in highly purified or crystalline form (3-5, 12, 13, 17, 18). While it is suspected that some of these viruses are transmitted by insects, the specific insect vectors are not known. Potato virus Y and Hyoscyamus virus 3, which are known to be transmitted by insects, were purified by Bawden and Pirie by chemical means (6), but the products were considered impure and largely inactivated. It seemed desirable, therefore, to attempt the isolation in a highly purified and active form of a virus known to be transmitted by a specific insect vector and to compare its properties with those of previously isolated viruses.

Alfalfa-mosaic virus (*Marmor medicaginis* H.)¹ is transmitted by the pea aphid, *Macrosiphum (Illinoia) pisi* Kalt. (20). The virus can also be transmitted mechanically, and produces local lesions on some varieties of *Phaseolus vulgaris* L., making it possible to obtain quantitative estimations of virus activity. Since the virus is rather unstable, isolation was attempted by means of differential centrifugation at low temperature, a method that has proved well suited for such viruses (18). The results of the application of this method to alfalfa-mosaic virus are here presented, together with a description of some of the properties of the purified material.

EXPERIMENTAL

The virus used is believed to be identical with or a closely related strain of the virus designated by Zaumeyer as alfalfa-mosaic virus 1 (21). Virus activity was determined by the half-leaf method, as described by Loring for tobacco-mosaic virus (11), using *Phaseolus vulgaris* var. Early Golden Cluster as the test plant. Unless otherwise stated, all inoculations were made in 0.1 M phosphate buffer at pH 7.1. Plants of Holmes' necrotic-type tobacco (7), derived from Turkish tobacco (*Nicotiana tabacum* L.), were inoculated while young and harvested 10 to 12 days later, since, at greater intervals after inoculation, the plants contain much smaller amounts of virus (14).

¹ Nomenclature presented in *Handbook of Phytopathogenic Viruses*, by Francis O. Holmes, Burgess Publishing Company (Minneapolis). 1939.

Extraction of Virus

If such plants are ground and pressed without altering the pH of the juice, only a portion of the virus contained in them is removed (14). If sodium hydroxide or dipotassium phosphate is added to the ground plant material in quantities such that the juice will be at about pH 7, the amount of virus obtained is more than doubled. Juice from unfrozen plants is 2 to 4 times as active as that from plants frozen at -14° C. and nearly twice as active as that from plants frozen rapidly with solid carbon dioxide. The extracted juice (at about pH 7) may be frozen by the latter method without appreciably affecting the activity, while freezing at -14° C. results in a reduction of 20 to 30 per cent. The losses caused by freezing are probably mechanical in nature, for, as will be shown later, freezing has little or no effect on the virus when purified. Centrifugation on a Swedish angle centrifuge or filtration through celite (Hyflo Super-Cel) does not remove all of the chlorophyll. Filtration through a finer grade of celite (Standard Super-Cel) that removes all chlorophyll also removes about 90 per cent of the virus.

In the method generally used, the harvested plants were cooled to about 4° C., then put through a meat grinder. Three per cent by weight of dipotassium phosphate in the form of a 50 per cent aqueous solution was added to the pulp, which, after thorough mixing, was allowed to stand at 4° C. for about 1 hour. The juice was removed by pressing through bandage gauze, placed in round-bottom flasks or lusteroid centrifuge tubes that were stoppered and placed at -14° C. or in solid carbon dioxide. When completely frozen, the juice was thawed by rotating the flasks under cold tap water or by centrifuging at room temperature. The juice was then centrifuged on a Swedish angle centrifuge at 4° C. or filtered through celite at 4° C.

Ultracentrifugation

The clarified juice was ultracentrifuged at 0° to 5° C. in a manner similar to that described by Stanley for the isolation of tobacco-ringspot virus (18). About $1\frac{1}{2}$ hours at 30,000 r.p.m. in a field of approximately 60,000 times gravity were required to sediment all of the virus. The pellets were dissolved in 0.1 M or 0.01 M phosphate buffer at pH 7.1, or in one of the foregoing buffers containing 0.5 mg. cysteine per cc., or in such other solvents as will be mentioned later. The insoluble material was removed by low-speed centrifugation at 4° C. for 30 to 60 minutes, and the supernatant liquid containing the virus was again subjected to ultracentrifugation. The process of high-then low-speed centrifugation was repeated 2 or 3 times, but in practically all cases the virus was contaminated by a green material, which contained phosphorus, nitrogen, and carbohydrate. It was found possible to remove this material by centrifuging at 30,000 r.p.m. for 1 minute. The green mate-

rial was sedimented and the supernatant liquid, containing practically all of the virus, was decanted into clean tubes and ultracentrifuged for $1\frac{1}{2}$ hours. The process of 3 or 4 cycles of alternate high- and low-speed centrifugations, with two short-time high-speed runs resulted in the isolation of a high molecular weight protein, free of the green material and possessing virus activity. The purified material was invariably light-brown, but repeated ultracentrifugation did not appreciably reduce the amount of brown pigment.

Chemical Composition and Yield of Purified Virus

In table 1 are listed data on the yields and analyses of purified preparations obtained by different procedures. The samples used for analysis were dialyzed for 24 hours at 4° C. against distilled water adjusted to pH 7 with sodium hydroxide and protected from carbon dioxide with a soda lime tube. This procedure was necessary to prevent the isoelectric precipitation of the virus by dissolved carbon dioxide. Dialysis under these conditions does not appreciably affect virus activity. In 7 tests, the dialyzed preparations averaged 89 per cent as active as controls kept at 4° C. The phosphorus and carbohydrate contents of the several preparations varied considerably, the averages being 1.58 per cent and 8.5 per cent, respectively. The true phosphorus content is probably more nearly 1.3 per cent, a value approached by several of the samples. Those samples that contained the larger amounts of phosphorus were possibly incompletely dialyzed or insufficiently purified. The latter possibility is suggested by the tendency of the 4-times-centrifuged samples to be lower in both phosphorus and carbohydrate than the corresponding samples that were centrifuged only 3 times. On the assumption that the phosphorus and carbohydrate are present as nucleic acid, the carbohydrate:phosphorus ratio was high in nearly all samples. The excess carbohydrate was probably an impurity and was quite possibly associated with the brown pigment. A highly active preparation was well dialyzed against water, then frozen and dried. It contained 53.87 per cent carbon, 6.69 per cent hydrogen, 16.25 per cent nitrogen, 1.44 per cent phosphorus, and 0.65 per cent sulphur. The analyses are those that would be expected for a nucleoprotein containing about 14–16 per cent nucleic acid and a few per cent extraneous carbohydrate.

Apparently, some loss of activity occurred during the purification process, for the activity of the final product usually accounted for but 20 to 40 per cent of that of the starting material (Table 1, column 5). These values were obtained by direct comparison of the activity of a 1:10 dilution of the juice with that of known dilutions of the purified material. Since the volumes of the juice and of the purified preparation were recorded, the percentage recovery could be calculated. It appears likely that much of the loss in activity was due to actual loss of virus and not to inactivation, for during the procedure

comparatively large amounts of insoluble precipitates, known to have absorbed appreciable quantities of virus, were discarded. It is possible, however, that some inactivation did occur and that the material isolated contained partially inactivated virus.

TABLE 1

Yields and Analyses of Ultracentrifugally Isolated Preparations of Alfalfa-Mosaic Virus

| Nature of starting material | Solvent used | No. of centrifugation cycles | Yield | | Phosphorus ^a | Carbohydrate ^a | Carbohydrate: P ratio |
|-----------------------------------------|-----------------------------------------|------------------------------|------------------------|----------|-------------------------|---------------------------|-----------------------|
| | | | Virus per cc. of juice | Activity | | | |
| Unfrozen juice | 0.1 M phosphate | 3 ^b | 0.14 | 30 | 1.70 | 10.8 | 6.4 |
| | | 3 ^b | — | — | 1.96 | 7.9 | 4.0 |
| | | 4 ^b | — | — | 1.77 | 7.4 | 4.2 |
| | 0.1 M phosphate + cysteine ^c | 3 | 0.19 | 17 | 1.38 | 7.4 | 5.4 |
| Juice frozen with solid CO ₂ | 0.1 M phosphate | 3 | 0.43 | 43 | 1.24 | 6.5 | 5.2 |
| | | 3 | 0.20 | 23 | 1.52 | — | — |
| | | 3 | 0.21 | 20 | 1.76 | 11.2 | 6.4 |
| | 0.1 M phosphate + cysteine ^c | 3 | — | — | 1.66 | 9.3 | 5.6 |
| | | 3 | 0.20 | 16 | 1.52 | 6.6 | 4.3 |
| | 0.1 M phosphate | 3 ^b | 0.09 | 12 | 1.85 | 10.0 | 5.4 |
| Juice frozen at -14°C. | 0.1 M phosphate + cysteine ^c | 3 | 0.32 | 45 | 1.32 | 7.9 | 6.0 |
| | | 3 | 0.17 | — | 1.35 | 7.8 | 5.8 |
| | | 3 ^b | 0.14 | — | 1.73 | 10.2 | 5.9 |
| | 0.1 M citrate + cysteine ^c | 4 ^b | — | — | 1.61 | 8.9 | 5.5 |
| | | 3 | 0.21 | — | 1.29 | 6.8 | 5.3 |

^a The values in these columns were calculated from analyses for nitrogen, carbohydrate, and phosphorus and are based on the assumption that the nitrogen content of the samples was 16 per cent. Phosphorus was determined by the method of King (9) and carbohydrate with orcinol and sulphuric acid (19).

^b Isolated from the same batch of plants.

^c 0.5 mg. per cc.

The results presented in table 1 indicate that neither the method of preparing the juice nor the solvent used for dissolving the pellets had much effect on either the yield or the composition of the purified protein. There was, however, an effect on other properties. The use of cysteine generally resulted in preparations that possessed more activity and a higher degree of homogeneity when examined in the ultracentrifuge. The green contaminant

was most easily removed when the juice was frozen at -14° C. In other experiments very poor yields of protein, badly contaminated with the green material, were obtained when the juice was not frozen. When distilled water containing cysteine and adjusted to pH 7 was used, the isolated protein was mostly inactive and quite heterogeneous, while the use of distilled water alone resulted in very poor yields. The use of citrate buffer resulted in a preparation about 70 per cent as active as one prepared from the same material with phosphate buffer.

It may be calculated from the data in the 4th and 5th columns of table 1 that the expressed juice of diseased plants contains about 1 mg. of virus per cc. A similar value also is indicated by the fact that juice diluted 1:10 produces about the same number of lesions on bean leaves as does the purified material at a concentration of 10^{-4} gm. per cc. This amount is present only during the winter months, as indicated by the fact that much smaller yields are obtained during the summer. During the latter season it is also much more difficult to remove the green contaminant, and the purified samples are high in phosphorus and carbohydrate.

Preliminary Concentration of the Virus

Attempts were made to effect a preliminary concentration of the virus, so that the amount of ultracentrifuging could be reduced. Since it was found that a single precipitation with 30 per cent ammonium sulphate in the cold had little if any effect on the activity of the virus, this salt was used to precipitate the virus from the juice. The precipitated virus was dissolved in 0.1 M phosphate buffer containing 0.5 mg. cysteine per cc. and equal in volume to $\frac{1}{10}$ that of the original juice. This was then subjected to 3 centrifugation cycles in the usual manner. In a control experiment, virus was isolated from some of the same juice by the usual 3 centrifugation cycles, using 0.1 M phosphate buffer containing cysteine as solvent for the pellets. The hydrogen-ion concentration of still another portion was adjusted to pH 4.6 with acetic acid. The precipitate was removed by centrifugation and treated exactly the same as the ammonium-sulphate-precipitated virus. All operations were carried out at 4° C. The preparations obtained were compared with respect to activity, then dialyzed at 4° C. for 24 hours, analyzed, and again compared for activity. As may be seen from the results given in table 2, both methods of concentration resulted in smaller yields and less active preparations, although that treated with ammonium sulphate was nearly as active as that isolated by ultracentrifugation alone. The sample precipitated with acetic acid was much lighter in color than any other preparation so far obtained, while the one precipitated with ammonium sulphate was much darker and less homogeneous, as indicated by the fact that continued low-speed centrifugation continued to remove material. The significance of the differences in composition is lessened

by the unaccountably high phosphorus content of the control. Because of the effect upon activity, these methods were not used.

It was found possible to obtain some concentration by simply freezing the juice in lusteroid tubes, then centrifuging at room temperature until thawed, but no longer, and siphoning off the upper $\frac{2}{3}$ of the supernatant liquid. The virus, together with most of the pigment, was found to be in the lower third. This method of obtaining approximately a threefold concentration of virus has been used rather extensively. The success of the

TABLE 2
Yields, Relative Activities, and Analyses of Purified Virus Preparations Obtained by Ultracentrifugation following Preliminary Concentration

| Method of concentration | No. of centrifugation cycles | Yield per cc. of juice | Relative activity ^a | | Phosphorus ^{b,c} | Carbohydrate ^b |
|-------------------------------------------------------------|------------------------------|------------------------|--------------------------------|-----------------------------|---------------------------|---------------------------|
| | | | Before dialysis | After dialysis ^a | | |
| | | mg. | per cent | per cent | per cent | per cent |
| (NH ₄) ₂ SO ₄ 30 per cent | 3 | 0.14 | 68 | 63 | 1.82 | 6.8 |
| Acetic acid pH 4.6 | 3 | 0.10 | 41 | 36 | 1.34 | 5.6 |
| Ultracentrifugation alone | 3 | 0.24 | 100 | 88 | 2.06 | 7.6 |

^a The figures in these columns were obtained by dividing the number of lesions produced when the designated sample was rubbed on 24 or more half leaves of *Phaseolus vulgaris* var. Early Golden Cluster by the number produced when a solution of the control of the same concentration was rubbed on the opposite halves, and multiplying by 100. The control used in each case was the solution of virus isolated entirely by ultracentrifugation and stored at 4°C.

^b The values in these columns were calculated from analyses for nitrogen, carbohydrate, and phosphorus and are based on the assumption that the nitrogen content of the sample is 16 per cent. Phosphorus was determined by the method of King (9) and carbohydrate with orcinol and sulphuric acid (19).

^c The samples upon which these analyses and activity measurements were made were dialyzed in a rocking dialyzer at 4°C for 24 hours against 50 liters of distilled water at about pH 7.

method is probably due to the fact that the pure ice crystals, which concentrate at the top, are the last to thaw.

General Properties

Solutions of the virus containing 5 to 10 mg. per cc. are only slightly opalescent and do not exhibit stream double refraction. The jelly-like pellets obtained by ultracentrifugation are only slightly birefringent. This may be due to the photoelastic effect, for it is increased by creating a strain in the pellet by pressing with a needle. Unlike tobacco-mosaic virus, samples of alfalfa-mosaic virus, when brought to the isoelectric point, do not form crystals nor is there any indication of a sheen. It appears likely, therefore, that the particles are essentially spherical in shape. Ultracentrifugation studies indicated that the virus has a sedimentation constant of approximately 74×10^{-13}

cm./sec. in unit centrifugal field (10). The preparations did not possess the same degree of homogeneity with respect to rate of sedimentation or electrophoretic mobility as tobacco-mosaic virus. The results indicated that each preparation consisted of a continuous distribution of particle sizes with a limited range about a modal value. The density of the protein, measured by means of a pycnometer, was found to be 1.48. Assuming a spherical shape, alfalfa-mosaic virus protein therefore has a molecular weight of about 2.1×10^6 and a diameter of 16.5 μ . The isoelectric point estimated from the point of minimum solubility was pH 4.6 (10).

In the absence of salt, the protein is soluble in 90 per cent alcohol and to a limited extent in hot 5 per cent trichloroacetic acid. However, if salt is present, the virus is insoluble in both solvents. Attempts to crystallize the virus by the use of ammonium sulphate or by dialysis were unsuccessful.

All of the more carefully prepared samples had about the same specific activity, i.e., a concentration of 10^{-4} g. per cc. caused 20 to 40 lesions per half leaf and one of 10^{-5} g. per cc. an average of about 1 lesion. Several of the preparations were rubbed on leaves of *Nicotiana glutinosa* L. and found to be free of tobacco-mosaic virus. When the purified protein was rubbed on tobacco leaves, typical symptoms of alfalfa mosaic were produced.

Isolation of Nucleic Acid

Since alfalfa-mosaic virus appeared to be a nucleoprotein, an attempt was made to isolate nucleic acid from it by the method of Johnson and Harkins (8) for the isolation of yeast nucleic acid. In one experiment, sufficient concentrated sodium hydroxide to make a 5 per cent solution was added to 4 cc. of a solution at 0° C. containing 40 mg. of protein. After standing at 0° for 1 minute, the solution was made acid to litmus by the addition of glacial acetic acid and the precipitated protein removed by centrifugation and then washed once with a small volume of water. The supernatant liquid plus the wash water was acidified to Congo red with concentrated hydrochloric acid and then treated with an equal volume of alcohol. The precipitated nucleic acid was removed by centrifugation and, after being washed 3 times with alcohol and dried *in vacuo* over phosphorus pentoxide at 110° C., weighed 3.1 mg. The material was dissolved in dilute sodium hydroxide and, upon analysis, found to contain 14.2 per cent nitrogen by the Kjeldahl method, 9.8 per cent phosphorus by the method of King (9), and 41 per cent carbohydrate by the orcinol method (19). The isolation experiment was repeated, using 161.5 mg. protein in 15 cc., and 14.5 mg. nucleic acid were obtained. Upon combustion, the latter was found to contain 34.47 per cent carbon, 3.73 per cent hydrogen, 8.34 per cent phosphorus, and 15.41 per cent nitrogen. The material is similar to yeast nucleic acid in elementary composition and, like it, gives negative Dische and Feulgen reactions. It may be concluded that alfalfa-mosaic virus

is a nucleoprotein containing a nucleic acid of the type obtained from yeast and several other viruses. Only about 60 per cent of the phosphorus present in the protein was isolated in the form of nucleic acid, but, since viruses differ in the ease with which nucleic acid is split off with sodium hydroxide (18), it is possible that the hydrolysis may have been incomplete. The fact that the

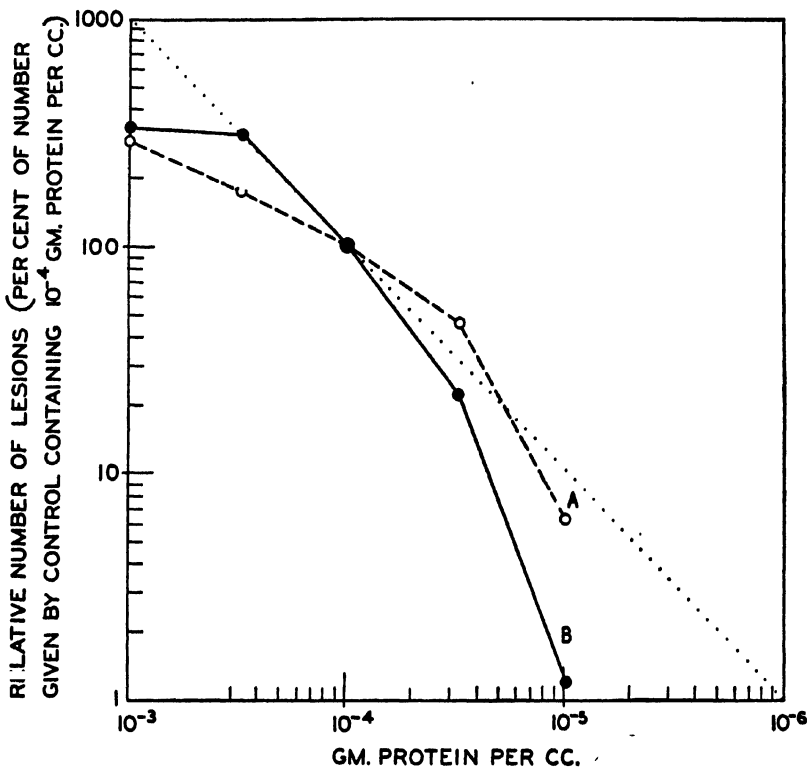


FIG. 1. Dilution curve of purified alfalfa-mosaic virus protein. Curves A and B are the dilution curves of the sample in 0.01 M and 0.1 M phosphate buffers at pH 7.1, respectively. The dotted line represents a theoretical dilution curve of slope 1.

protein component obtained in the second isolation experiment was found to contain 0.2 per cent phosphorus and 2 per cent carbohydrate is in accordance with this viewpoint.

Dilution Curve

The dilution curves of a number of preparations in different solvents were studied so that optimum conditions for activity measurements could be determined. Several different concentrations of virus protein were prepared in

a given solvent and their activities compared with that of a control containing 10^{-4} g. per cc. in the same solvent. The dilution curves of a typical preparation in 0.1 M and 0.01 M phosphate buffers are given in figure 1. The results are expressed as relative number of lesions given by each dilution, taking the number given by the control as 100 per cent. In 0.1 M phosphate buffer, the number of lesions is proportional to the virus concentration within a limited range. At greater concentrations, as is the case with many viruses, the dilution curve has a slope of less than unity. However, at dilutions higher than 5×10^{-4} g. per cc., the slope becomes much greater than unity. This last effect has been observed also with juice containing alfalfa-mosaic virus (14) and with certain other viruses (2). The dilution curves of several samples of alfalfa-mosaic virus protein were determined and all were of the same gen-

TABLE 3
Accuracy of Activity Measurements

| Virus concentration | Relative virus concentration | Relative number of lesions ^a |
|----------------------|------------------------------|-----------------------------------------|
| g./cc. | per cent | per cent |
| 1.4×10^{-4} | 140 | 124.0 |
| 1.2×10^{-4} | 120 | 117.5 |
| 1.0×10^{-4} | 100 | 83.2 |
| 0.8×10^{-4} | 80 | 71.0 |
| 0.6×10^{-4} | 60 | 63.8 |
| 0.4×10^{-4} | 40 | 34.8 |

^a The figures in this column were obtained by dividing the number of lesions produced when the designated sample was rubbed on 24 or more half leaves of *Phaseolus vulgaris* var. Early Golden Cluster by the number produced when a solution containing 10^{-4} g. of virus per cc. was rubbed on the opposite half leaves, and multiplying by 100. All inoculations were made in 0.1 M phosphate at pH 7.1.

eral shape. In 0.01 M phosphate buffer the dilution curve has a slope of less than one, except at very high dilutions. Such a solvent was considered unsuited for activity measurements; hence, 0.1 M phosphate buffer was used in nearly all cases. An attempt was always made to inoculate at a concentration not far from 10^{-4} g. per cc., for in that region the number of lesions is approximately proportional to the virus concentration. The accuracy to be expected when these precautions are taken is indicated by the data in table 3. Samples differing in protein concentration by 20 per cent were compared with a common control containing 10^{-4} g. per cc. by the method previously described. In 4 of the tests the concentrations estimated from activity data did not differ by more than 10 per cent from the actual concentration, and in the other 2 tests the difference was less than 20 per cent.

The total number of lesions produced by a given sample is also influenced by the solvent (Table 4). Hence, when the relative activity of 2 or more

samples was estimated, care was taken that they were in the same solvents. The specific activity of purified preparations of alfalfa-mosaic virus appears to be considerably less than that of other viruses isolated in this laboratory. This is not sufficient reason for believing, however, that the virus was inactivated during the isolation procedure, as was thought to be the case with potato virus Y and Hyoscyamus virus 3 (6). If any inactivation occurred during the purification of alfalfa-mosaic virus, it could not have been great, for in some cases approximately half of the total activity represented by the starting material was recovered in the isolated nucleoprotein; hence, the loss was no greater than might be expected to occur due to mechanical reasons. It is

TABLE 4

Effect of Solvent on Number of Lesions Produced by Purified Alfalfa-Mosaic Virus

The figures represent the numbers of lesions per half leaf produced on 24 or more half leaves of *Phaseolus vulgaris* var. Early Golden Cluster by virus preparations of the designated concentration dissolved in the designated solvent. The samples paired vertically were rubbed on opposite half leaves. All solutions were at pH 7.1.

| Solvent | Virus concentration | |
|------------------|-------------------------|-------------------------|
| | 10 ⁻⁴ g./cc. | 10 ⁻⁶ g./cc. |
| 0.1 M phosphate | 58.5 | 0.3 |
| 0.01 M phosphate | 102.9 | 4.7 |
| 0.1 M phosphate | 25.5 | 0.5 |
| 0.01 M phosphate | 30.9 | 3.2 |
| 0.1 M phosphate | 13.7 | — |
| 0.1 M citrate | 4.1 | — |
| 0.01 M phosphate | 17.6 | — |
| 0.01 M citrate | 6.5 | — |

possible that the specific activity may appear low owing to the host used for activity determinations, for the amount of material required to cause infection when rubbed on plants varies considerably with the species and with the variety of the plant.

pH Stability

To aid in the selection of the optimum conditions for isolation and for storage, the stability of the virus at different hydrogen-ion concentrations was determined. Five-cc. portions of a well-dialyzed virus solution containing 2×10^{-3} g. per cc. were cooled to 4° C. and added to equal quantities of cold 0.075 M phthalate-phosphate-borate buffer previously adjusted to different hydrogen-ion concentrations with sodium hydroxide or hydrochloric acid. The hydrogen-ion concentrations were then determined at room temperature

and the samples were stored at 4° C. Immediately after mixing and at intervals samples were removed, diluted 1:10 with 0.1 M phosphate buffer at pH 7.1, and used for activity measurements. One of the samples, that at pH 7.1, was taken as the control and each sample was compared with it. The hydrogen-ion concentration of the solutions used for inoculation did not differ by more than 0.1 pH unit. The results are presented in figure 2. The virus is most stable at about pH 7 and is fairly stable over the range pH 5.5 to 7.7.

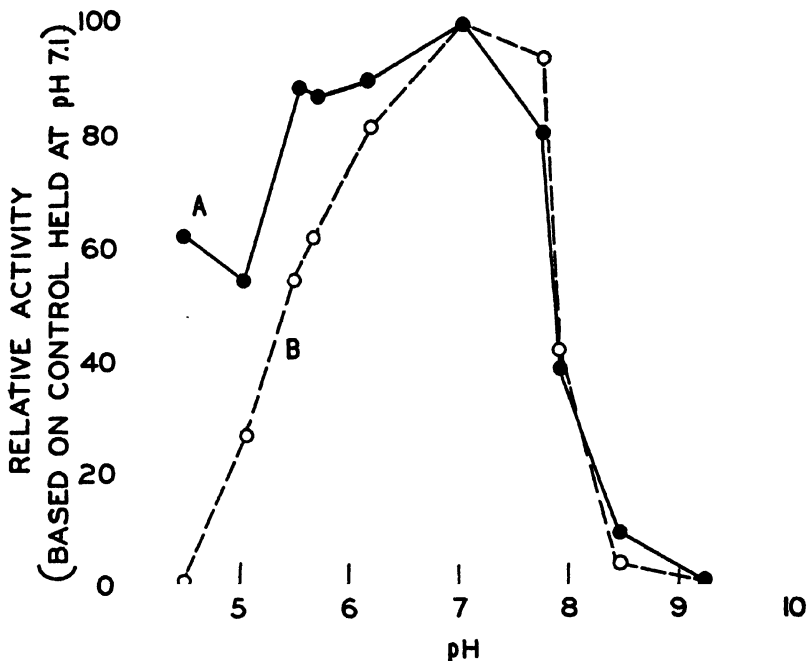


FIG. 2. Stability of purified alfalfa-mosaic virus at 4° C. at different hydrogen-ion concentrations. Curves A and B represent the relative activity of the samples after standing at the various pH values for 20 hours and 120 hours, respectively. They were adjusted to pH 7.1 immediately before being used for inoculation.

This is a narrower pH stability range than has been reported for other plant viruses. The virus is much more stable at pH 7 than it is at its isoelectric point, pH 4.6. The experiment was repeated twice, once with purified virus and once with partly purified virus. Comparable results were obtained.

Effect of Storage

In figure 2, the results are presented as relative changes in activity, and, consequently, give no indication of the stability of the control. Since the

actual number of lesions produced by a sample is affected by many environmental factors, the average number of lesions produced by a sample on succeeding days is not a good measure of changes in the activity of the sample. If the virus is not affected by freezing and thawing, changes in the activity of a sample could be followed by comparing with a control kept in the frozen state. It appeared desirable, therefore, to determine the effect of freezing and thawing on the virus under different conditions.

Samples containing 1 mg. of alfalfa-mosaic virus per cc. in nutrient broth, in 0.1 M phosphate buffer, and in water were frozen rapidly with carbon dioxide and left frozen for 20 hours. They were then thawed, diluted with 0.1 M phosphate, and compared for activity with similar samples stored at 4° C. The samples that had been frozen were 80, 86, and 85 per cent as active, respectively, as the unfrozen controls. Corresponding samples placed in a room held at -14° C. for the same length of time were 79, 89, and 69 per cent as active, respectively, as the controls. The experiment was repeated and similar results were obtained. In further tests, samples in 0.1 M phosphate buffer that were frozen for short periods of time in carbon dioxide averaged 94 per cent as active as the controls. While most of these differences are not significant when considered individually, the fact that they are all in the same direction indicates that a small amount of inactivation may result from freezing and thawing. Alfalfa-mosaic virus, therefore, differs from tobacco-ringspot virus (18), which is denatured by freezing in water. When solutions of alfalfa-mosaic virus are frozen and thawed, there is no indication of denaturation, for no insoluble precipitate is formed. However, unless samples are sealed in glass, those frozen by means of carbon dioxide and containing no buffer may absorb sufficient carbon dioxide to cause the isoelectric precipitation of the protein.

A solution of purified virus containing 1 mg. per cc. in 0.1 M phosphate at pH 7.1 was prepared, and, since freezing and thawing have only a slight effect on the virus, aliquot portions to be used as controls were sealed in glass and stored in solid carbon dioxide. Others were stoppered and stored at 4° C., and, since, even at that temperature, some bacterial action occurs, toluene was added to some. At intervals, samples were removed and used for activity comparisons. In one experiment there were no significant differences in the activity of the several lots up to a period of 4 days. In another experiment there were no differences apparent in 3 days; but, in 12 days, the samples stored without toluene at 4° C. were 44 per cent as active as the control stored in the frozen state, while those to which toluene was added were 65 per cent as active as the controls. After a period of 27 days the percentages were 44 and 62 without and with toluene, respectively. The data indicate that the purified virus is relatively stable at 4° C. in 0.1 M phosphate buffer at pH 7.1, but that inactivation does occur over a long period of time.

The addition of toluene has a slight effect in preserving virus activity. It is possible that the activity of the frozen samples decreases with time, but this does not appear probable, for the samples kept frozen for 27 days still produced about as many lesions per half leaf as they did when the experiment was started, indicating that no great change in activity had occurred. Although the virus is fairly stable at 4° C., inactivation proceeds rather rapidly at higher temperatures. A sample in 0.1 M phosphate buffer and containing toluene was held at 24° C. and lost 72 per cent of its activity in 1 day, 74 per cent in 2 days, and 95 per cent in 4 days.

A comparison was made of the stability of the virus in distilled water, and in 0.01 M and 0.1 M phosphate buffers at pH 7. After 6 days at 4° C., the sample in 0.1 M phosphate buffer was 59 per cent as active as that in 0.01 M phosphate buffer and about twice as active as that stored in water. After 28 days, the sample in 0.1 M phosphate was only 19 per cent as active as that in 0.01 M phosphate and 52 per cent as active as that in water. Repetition of the experiment gave comparable results. Hence, the virus is more stable in 0.01 M phosphate buffer than it is in 0.1 M phosphate. The behavior in the absence of buffer is somewhat erratic, probably because of changes in pH brought about by absorption of carbon dioxide. Activity is lost much more rapidly in juice than in purified solutions. A sample of juice at pH 7 stored at 4° C. lost 52 per cent of its activity in 3 days and 87 per cent in 7 days, when compared to a portion of the same juice stored in the frozen state.

Effect of Reducing Substances on the Virus

Since cysteine and other reducing agents have a stabilizing effect on some plant viruses (1, 18), it appeared desirable to determine their effect on alfalfa-mosaic virus. In one experiment conducted at 4° C., pellets obtained by ultracentrifugation of infectious juice were dissolved in 0.1 M phosphate buffer at pH 7.1 equal in volume to $\frac{1}{4}$ that of the original juice. In a second experiment conducted at 24° C., a solution of purified virus containing 1 mg. per cc. was used. Solutions of cysteine, sodium sulphite, and sodium hydrosulphite were adjusted to pH 7 with sodium hydroxide and then added to different portions of the virus solution. At intervals, samples were diluted 10 times with 0.1 M phosphate buffer and compared for activity with controls treated in exactly the same manner, except that water was added instead of the solutions of reducing agents. It may be seen from table 5 that cysteine has no effect on purified virus but that it tends to stabilize partially purified virus. Because of these results, cysteine was usually used in the purification process. On the other hand, sulphite and hydrosulphite inactivate the virus. The cysteine probably protects the virus from the inactivating effect of some substance that is eliminated by the purification process.

TABLE 5

Effect of Reducing Substances on Alfalfa-Mosaic Virus

[The figures represent the number of lesions per half leaf produced when 24 or more half leaves of *Phaseolus vulgaris* var. Early Golden Cluster were rubbed with a 1:10 dilution of the indicated sample.]

| Type of preparation | Hours | Temperature | Cysteine ^a | | Sodium sulphite ^a | | Sodium hydro-sulphite ^a | |
|-----------------------------|-------|-------------|-----------------------|---------|------------------------------|---------|------------------------------------|---------|
| | | | Sample | Control | Sample | Control | Sample | Control |
| | | °C. | | | | | | |
| Ultracentrifuged 1 time | 0 | 4 | 45.8 | 35.8 | 26.7 | 28.8 | 14.2 | 37.4 |
| | 14 | 4 | 15.7 | 15.3 | 15.8 | 14.6 | 14.2 | 17.4 |
| | 60 | 4 | 9.6 | 8.4 | 1.4 | 10.9 | 2.8 | 10.0 |
| | 120 | 4 | 24.3 | 13.3 | 0.5 | 10.8 | 1.0 | 13.4 |
| Ultracentrifuged 4 times | 0 | 24 | 13.4 | 22.7 | 13.8 | 8.7 | — | — |
| | 24 | 24 | 1.9 | 2.6 | 0.2 | 5.3 | — | — |
| | 48 | 24 | 1.1 | 2.4 | 0.1 | 4.0 | — | — |
| | 96 | 24 | 0.1 | 0.3 | 0.2 | 1.1 | — | — |

^a 1 mg. per cc.

TABLE 6

Rate of Inactivation of Alfalfa-Mosaic Virus by Trypsin

[The reaction mixtures consisted of 4.3 mg. virus and 0.043 mg. trypsin per cc. in 0.1 M phosphate buffer at pH 7.1.]

| Temperature | Time | Lesions | | Relative activity of trypsin-treated sample |
|-------------|------|------------------------------|----------------------|---------------------------------------------|
| | | Trypsin-treated ^a | Control ^a | |
| | hr. | | | per cent |
| 25°C. | 0 | 68.4 | 46.7 | 147 |
| | 1 | 23.0 | 27.2 | 85 |
| | 3 | 5.8 | 14.9 | 39 |
| 19°C. | 0 | 58.1 | 41.3 | 141 |
| | 1 | 32.6 | 37.1 | 88 |
| | 3 | 1.7 | 14.5 | 11 |
| | 6 | 3.3 | 45.8 | 7 |
| | 24 | 1.1 | 8.3 | 13 |

^a The figures in these columns represent the number of lesions per half leaf produced when the designated sample and the corresponding control were rubbed on opposite half leaves of 24 or more leaves of *Phaseolus vulgaris* var. Early Golden Cluster. The samples were diluted 1:10 with 0.1 M phosphate buffer at pH 7.1.

Effect of Trypsin

Since trypsin has been successfully employed in the purification of tobacco-mosaic virus and its strains (3, 15), the possibility of using it to remove the brown coloring matter associated with alfalfa-mosaic virus was investigated.

Crystalline trypsin, kindly supplied by Dr. M. Kunitz, was added to a solution of alfalfa-mosaic virus in 0.1 M phosphate buffer at pH 7.1 at a ratio of 1:100 and allowed to stand at room temperature. At intervals, samples were removed, diluted to a virus concentration of 10^{-4} g. per cc. with 0.1 M phosphate buffer, and compared for activity with a control treated in exactly the same manner, except that no trypsin was added. The results shown in table 6 indicate that the enzyme inactivates the virus, although there seems to be an initial activating effect. The inactivation is not merely an inhibition of the virus, as is the case with that of tobacco mosaic, for there is no immediate

TABLE 7

The Effect of Trypsin on Alfalfa-Mosaic Virus

[Each sample consisted of 14.4 mg. virus, corresponding to 2.31 mg. N, and 0.7 mg. trypsin dissolved in 3 cc. of 0.1 M phosphate buffer at pH 7.1.]

| Time | Temperature | Supernatant liquid from first ultracentrifugation | | N recovered as heavy protein | Relative activity of recovered protein ^a |
|----------------|-------------|---------------------------------------------------|------------------------|------------------------------|-----------------------------------------------------|
| | | Total N | Protein N ^b | | |
| <i>minutes</i> | <i>°C.</i> | <i>mg.</i> | <i>mg.</i> | <i>mg.</i> | <i>per cent</i> |
| Control | 4 | 0.36 | 0.27 | 1.05 | 100 |
| 0 | 24 | 1.11 | 0.58 | 0.75 | 85 |
| 30 | 24 | 1.57 | 0.90 | 0.52 | 35 |
| 60 | 24 | 1.72 | 1.02 | 0.42 | 10 |
| 120 | 24 | 1.87 | 0.96 | 0.34 | 1 |
| 240 | 24 | 2.00 | 0.99 | 0.24 | 1 |
| 240 | 4 | 1.41 | 0.75 | 0.66 | 53 |

^a The figures in this column were obtained by dividing the number of lesions produced when a solution of the designated sample containing 10^{-4} g. protein per cc. was rubbed on 24 or more half leaves of *Phaseolus vulgaris* var. Early Golden Cluster by the number produced when a solution of the control of the same protein concentration was rubbed on the opposite halves, and multiplying by 100.

^b Insoluble in 15 per cent trichloroacetic acid.

inhibition, and the inactivation is a function of time and proceeds at a measurable rate.

In order to establish more definitely the nature of the action of trypsin on alfalfa-mosaic virus, an experiment was conducted in which the rate of hydrolysis of heavy protein was also followed. To 3-cc. portions of alfalfa-mosaic virus containing 4.82 mg. protein per cc. in 0.1 M phosphate buffer at pH 7.1, 0.7-mg. portions of crystallized trypsin were added. Immediately after mixing and at intervals up to 4 hours, individual samples were diluted to 15 cc. with cold water to retard the reaction and ultracentrifuged at 0–5° C. for 1½ hrs. at 30,000 r.p.m. to separate the virus and enzyme. The upper half of the supernatant liquid was drawn off and analyzed for total and for protein nitrogen. The pellets were redissolved in 15 cc. of cold phosphate

buffer at pH 7 and the virus ultracentrifuged again. The second pellets were dissolved in a small volume of buffer and centrifuged at low speed. The supernatant liquids were then analyzed for nitrogen and their activities compared with that of a control treated in exactly the same manner, except that no trypsin was added. The samples were diluted to 10^{-4} g. per cc., assuming that all of the nitrogen present was protein nitrogen. The results are presented in table 7. It is evident that hydrolysis occurred, for the amount of heavy protein recovered decreased as the time of contact with the enzyme was lengthened and most of the nitrogen was recovered in the supernatant liquid. The rate of hydrolysis, as well as that of inactivation, was not so great at 4° C. as at the higher temperature. The nitrogen not sedimented in the ultracentrifuge was approximately equally divided between low molecular weight protein and non-protein nitrogen. As the reaction proceeded, there was a decrease not only in the amount of heavy protein recovered, but also in the activity of the material isolated. After 2 to 4 hours, the heavy protein that remained intact had lost about 99 per cent of its activity. The obvious conclusion is that inactivation proceeds at a faster rate than does the extensive cleavage of the heavy protein. The possibility exists, however, that the material isolated was not entirely protein. Nucleic acid might possibly be split off in fragments sufficiently large to be sedimented by the ultracentrifugation. The quantity isolated was too small to permit examination of its properties in detail, but it resembled the virus protein in appearance. If trypsin actually has an initial activating effect, as suggested by the data in table 6, it must be due to the presence of the enzyme and not to any permanent effect on the virus, for the activity of virus exposed to trypsin for a brief time and then separated from the enzyme did not differ significantly from that of the control.

SUMMARY

A high molecular weight nucleoprotein possessing the properties of alfalfa-mosaic virus, a virus known to be transmitted by a specific insect vector, has been isolated from diseased tobacco plants by means of differential centrifugation at a temperature of 0° to 4° C. The virus preparations contained about 15 per cent of a nucleic acid of the yeast nucleic acid type, about 0.65 per cent sulphur, and small amounts of extraneous carbohydrate. The virus appears to be essentially spherical in shape and has a specific gravity of 1.48, and a sedimentation constant of approximately 74×10^{-13} . It is the smallest plant virus that has been isolated, for the molecular weight and diameter, based on the foregoing constants, are 2.1×10^6 and $16.5 \text{ m}\mu$, respectively.

Freezing and thawing have only a slight effect on the purified virus, but similar treatment of diseased plant material results in a measurable loss of virus. The virus, when purified, is reasonably stable at 4° C., but is rapidly

inactivated at room temperature and is much less stable when in juice. Sodium sulphite and sodium hydrosulphite inactivate the virus, while cysteine has a stabilizing effect on partly purified preparations, but no effect on purified ones. The virus is slightly more stable in 0.01 M phosphate buffer than in 0.1 M phosphate buffer or in water. Toluene helps to preserve the virus.

The juice of diseased greenhouse-grown tobacco plants contains about 1 mg. of virus per cc. during the winter months and less in summer. When virus dissolved in 0.1 M phosphate buffer at pH 7 is rubbed on bean leaves, the number of lesions produced is proportional to the concentration virus over a limited range only. At concentrations less than 5×10^{-4} g. per cc., the number produced decreases more rapidly than does the virus concentration in the inoculum, and, at 10^{-5} g. per cc., few or no lesions result. The virus is inactivated and hydrolyzed by trypsin. The inactivation proceeds at a more rapid rate than does the extensive hydrolysis of the high molecular weight protein. In so far as has been determined, alfalfa-mosaic virus nucleoprotein does not differ fundamentally from other virus nucleoproteins that have been isolated and for which no specific insect vectors are known.

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THE CONCENTRATION OF ALFALFA-MOSAIC VIRUS IN TOBACCO PLANTS AT DIFFERENT PERIODS OF TIME AFTER INOCULATION¹

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It has been the practice in this laboratory to harvest virus-containing plants used as starting material in the purification of viruses about 4 weeks after inoculation. Attempts to isolate alfalfa-mosaic virus (*Marmor medicaginis* H.)² from tobacco plants that had been diseased for that length of time were, however, unsuccessful. Furthermore, it was found that the juice from such plants was practically noninfectious when rubbed on *Phaseolus vulgaris* L. var. Early Golden Cluster. This was contrary to the observation of Price (8), who obtained many lesions by rubbing with the juice of tobacco plants diseased with alfalfa mosaic. This contradiction suggested that the concentration of alfalfa-mosaic virus in tobacco plants might not be constant but might vary with the age of the plants. Consequently, a study was made of the virus content of diseased tobacco plants at different periods of time after inoculation. In the present paper are reported the results of the investigation, together with information on optimum conditions for the extraction of virus from the plant material and on dilution curves of the virus in juice from diseased plants.

EXPERIMENTAL

The virus used was obtained from W. C. Price and is believed to be identical with that designated by Zaumeyer as alfalfa-mosaic virus 1 (13). Holmes' necrotic-type tobacco (4), derived from Turkish tobacco (*Nicotiana tabacum* L.) and resistant to systemic infection by tobacco-mosaic virus, was used as host plant in most of the tests. This obviated possible contamination with tobacco-mosaic virus. The plants were grown in 4-inch pots in a greenhouse during the winter months. Twice weekly they were given light applications of a 0.06 per cent ammonium nitrate solution.

¹ A preliminary report of this work was presented before the American Phytopathological Society, at Columbus, Ohio, on December 28, 1939 (10).

² Nomenclature presented in *Handbook of Phytopathogenic Viruses*, by Francis O. Holmes, Burgess Publishing Company, Minneapolis, 1939.

Virus activity was determined by the half-leaf method, described by Loring for tobacco-mosaic virus (5), using *Phaseolus vulgaris* var. Early Golden Cluster as a test plant. For reasons to be discussed later, juice was diluted 1:10 with 0.1 M phosphate buffer at pH 7.1 before being used for activity measurements.

Extraction of Virus

It was found in 1936 (11) that the addition of disodium phosphate to the macerated pulp of frozen tobacco-mosaic-diseased plants resulted in an appreciably higher virus content in the extracted juice, and since then disodium or dipotassium phosphate has been widely used in the extraction of viruses. It appeared desirable, therefore, to determine the effect of this material when used in the extraction of alfalfa-mosaic virus from tobacco plants. Diseased plants were ground in a meat grinder and the pulp divided into 2 portions. To one portion was added 3 per cent by weight of dipotassium phosphate in the form of a 50 per cent aqueous solution. After thorough mixing, both portions were allowed to stand at 4° C. for 1 hour. The juice was then removed from each by pressing through bandage gauze. A solution of dipotassium phosphate, equivalent in amount to that added to the pulp of one sample, was added to the juice from the nontreated portion. The juices were then centrifuged at 3000 r.p.m. at 4° C. for 30 minutes, diluted 1:10 with 0.1 M phosphate buffer, and tested for virus activity. The juice from the sample to which phosphate was added before extraction produced 1311 lesions on 35 half leaves, and the other caused 553 lesions on the opposite halves of the same leaves. The hydrogen-ion concentrations of the two juices were pH 6.97 and 7.15, respectively, while the 1:10 dilutions used for inoculation were both at pH 7.1. Since the two samples differed only in the manner in which the juices were extracted, the data indicate that the addition of phosphate before extraction resulted in more than a 2-fold increase in the virus activity of the extracted juice.

The foregoing experiment was repeated with a few modifications to determine whether or not the enhanced extraction of virus was attributable to the change in pH or to a specific action of the phosphate. Ground plant material was divided into 3 portions, A, B, and C. Dipotassium phosphate was added to A as in the preceding experiment, while B received no treatment, and C was adjusted to pH 6.6 by the cautious addition of 2 N sodium hydroxide. After the pulp had stood at 4° C. for 1½ hours, the juice was pressed from each preparation and then centrifuged. The juices thus extracted were tested for virus activity at a dilution of 1:10 in 0.1 M phosphate buffer. It may be seen from the results presented in table 1 that the addition of alkali to the pulp was as effective as the addition of phosphate, for both A and C were about 6 times as active as B, and a direct comparison of A with C revealed no difference in activity. The addition of 3 per cent by weight of dipotassium phosphate

should result in a juice about 0.18 M with respect to phosphate, but, since appreciable quantities of insoluble phosphates are formed, the actual concentration would be less than 0.18 M. When such juice is diluted 1:10 with 0.1 M phosphate buffer, the phosphate concentration would be very near 0.1 M. As the samples differed only with respect to the method of extraction, it may be concluded that the addition of dipotassium phosphate before extraction of the juice results in an increase in the amount of virus extracted and that the effect is due to the increased pH and not to a specific effect of phosphate.

Dilution Curve

As an aid to the interpretation of activity data, dilution curves of the phosphate extract of tobacco plants diseased with alfalfa mosaic for different periods

TABLE 1
Extraction of Alfalfa-Mosaic Virus from Tobacco Plants

| Sample | Treatment of ground plant material | pH of juice | Concentration of phosphate buffer used for diluting | pH of inoculum | Lesions per half leaf ^a |
|--------|---------------------------------------------------------|-------------|-----------------------------------------------------|----------------|------------------------------------|
| A | K ₂ HPO ₄ added before extraction | 7.2 | 0.1 M | 7.1 | 25.0 |
| B | No treatment | 5.6 | 0.1 M | 7.1 | 3.4 |
| A | K ₂ HPO ₄ added before extraction | 7.2 | 0.1 M | 7.1 | 30.3 |
| C | NaOH added before extraction | 6.6 | 0.1 M | 7.1 | 30.0 |
| C | NaOH added before extraction | 6.6 | 0.01 M | 7.0 | 20.8 |
| B | No treatment | 5.6 | 0.01 M | 6.9 | 3.3 |

^a The samples paired vertically were rubbed on 24 or more opposite half leaves of *Phaseolus vulgaris* var. Early Golden Cluster.

of time were determined. Young tobacco plants were inoculated with alfalfa-mosaic virus, and, at intervals of 5, 12, and 32 days, 6 plants were harvested and ground. Three per cent by weight of dipotassium phosphate was added, and the juice was extracted and centrifuged as previously described. Several different dilutions were made with 0.1 M phosphate buffer at pH 7.1 and the dilutions were compared on opposite half leaves with a 1:10 dilution of the same juice. In figure 1, relative numbers of lesions are plotted against dilution on a logarithmic scale, with the number produced by the 1:10 dilution arbitrarily assigned the value of 100. The curves for the 5-, 12-, and 32-day samples are markedly similar. The upper portions of the curves are unusual, for in that region dilution results in increased lesion formation. This phenomenon is probably caused by an inhibitor in the juice, for solutions of purified alfalfa-mosaic virus do not behave in a similar manner (9). Black (2) obtained the same type of curve with solutions of tobacco-mosaic virus mixed with insect juices.

At intermediate dilutions the number of lesions is roughly inversely proportional to the dilution. The range over which this is true is not great and varies somewhat with the various juices, being greater in those containing

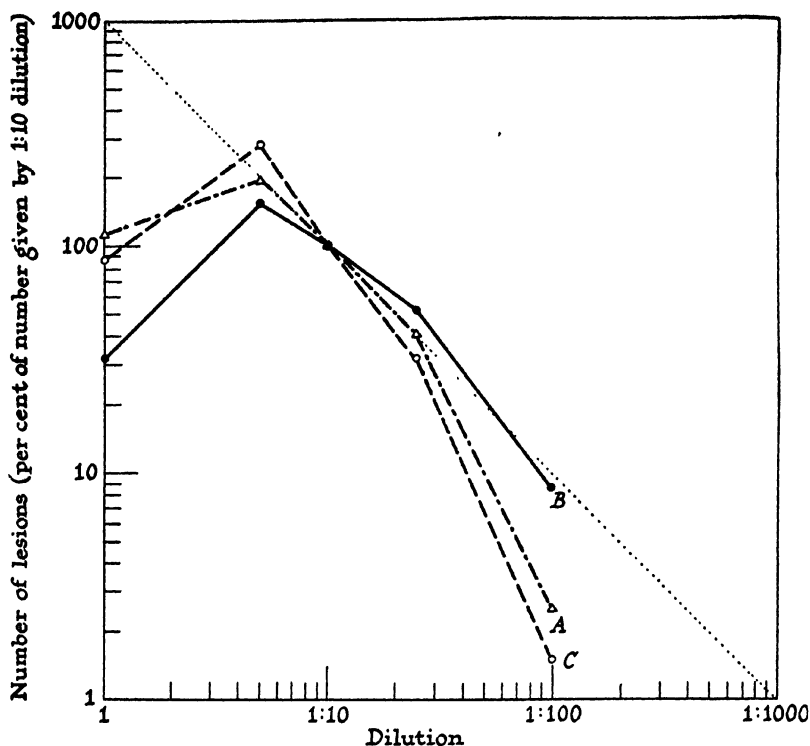


FIG. 1. Dilution curves of the juice obtained from tobacco plants diseased with alfalfa-mosaic virus for different periods of time. Juice was extracted after the addition of 3 per cent by weight of dipotassium phosphate and was approximately pH 7. The dilutions were made with 0.1 M phosphate buffer at pH 7.1. Each dilution of a given juice was rubbed on 24 or more half leaves of *Phaseolus vulgaris* var. Early Golden Cluster, and a 1:10 dilution of the same juice was rubbed on the opposite halves. Curves A, B, and C are the dilution curves of the juices obtained from plants diseased for 5, 12, and 32 days, respectively. The dotted line represents a theoretical curve of unit slope.

the most virus. Since the 1:10 dilution of each juice fell within this range, such a dilution was usually used in making activity measurements. At higher dilutions the dilution curve of alfalfa-mosaic virus again differs from that of most other viruses for which dilution-curve data are available, for the reduction in number of lesions is greater than the change in dilution, *i.e.*, the curve

has a slope greater than 1. The same reduction was noted with purified alfalfa-mosaic virus (9). It also has been reported for certain other viruses (1). In the present case it does not seem probable that the effect resulted from rapid inactivation of the virus in dilute solutions. Inoculations with the dilute solutions usually were made within 20 minutes after dilution; but, when greater periods of time elapsed, the several dilutions still possessed the same relative activities.

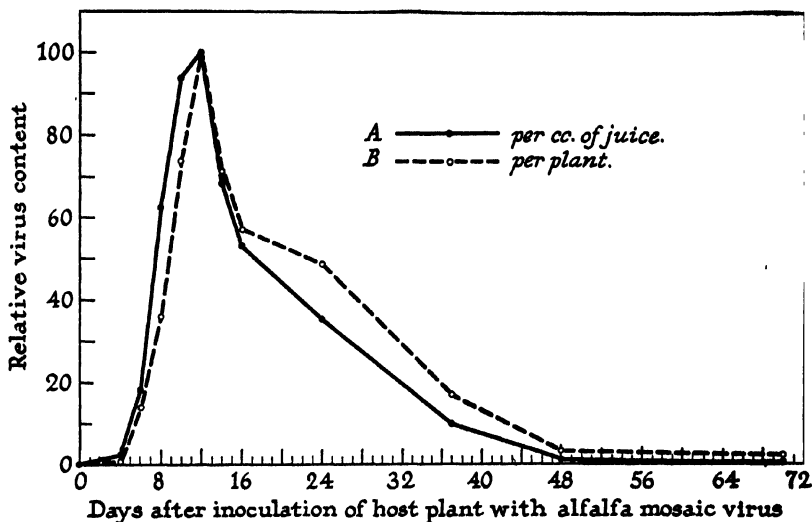


FIG. 2. Concentration of alfalfa-mosaic virus in tobacco plants diseased for different periods of time. Curve A represents the relative activity of the several juices. When the data were recalculated, taking into consideration the amount of juice obtained from each sample, the data represented by curve B were obtained. It is regarded as representing the relative amounts of virus present in the plants.

Virus Content of Plants at Intervals following Inoculation

Different lots of young tobacco (Holmes' necrotic-type) plants were inoculated with alfalfa-mosaic virus at intervals of 2 to 24 days. Each plant, at the time of inoculation, possessed 4 leaves over 4 inches in length. An attempt was made to select uniform plants and to inoculate with virus solutions of approximately the same virus concentration each time. At a later date, 6 plants from each lot were harvested and the juice was extracted from each by the method previously described in which dipotassium phosphate was used. Each sample of juice was diluted 1:10 with 0.1 M phosphate buffer and compared for activity with a common control which consisted of a comparable dilution of the juice from plants diseased for 12 days. The virus activity of the juice, represented by curve A in figure 2, increased rapidly from about the

4th to the 11th or 12th day after inoculation of the plants, then decreased rapidly until the lesion count was as low as 1 per cent of the maximum. The data, presented as relative numbers of lesions, are probably fair estimates of the relative virus contents of the samples. The data in figure 1 indicate that the 1:10 dilution is within the suitable range for study, even with the older juices, and that the dilution curve of the juice of plants diseased for 12 days approximates a straight line with a slope of unity until a dilution of 1:100 is reached. Hence, with samples producing over 10 per cent as many lesions as the control, the relative number of lesions would be proportional to the actual virus content. The virus content of those samples producing less than 10 per cent as many lesions as the control probably would be somewhat greater than the amount indicated. The experiment was repeated several times and similar results were obtained each time. In one experiment, instead of using one control for all tests, samples inoculated at successive intervals were compared, *i.e.*, the 4-day sample was compared with the 6-day, the 6-day with the 8-day, etc. In another experiment the various samples were diluted, so that each gave approximately the same number of lesions. The curve obtained in each case was similar to that shown in figure 2, except that samples from plants inoculated for long periods of time appeared to be slightly more active than corresponding ones in figure 2. In still another experiment, the several samples of juice were ultracentrifuged and the pellets obtained were dissolved in 0.1 M phosphate buffer equal in volume to the original juice. These partly purified preparations were used for inoculations. The fact that the results did not differ significantly from those obtained with the juices indicates that the differences noted with the latter did not result from the presence of varying quantities of an inhibitor. It is possible that such a substance would not be separated from the virus by ultracentrifugation. However, experiments with virus purified by ultracentrifugation (9) provided no evidence of the presence of an inhibitor associated with the purified material.

The low virus content of the juice of the older plants is not because of any diluting effect of growth, for the virus concentration changes at a much more rapid rate than does the size of the plants. When the data presented in curve A, figure 2, were recalculated, taking into consideration the increase in size of plants, curve B was obtained, representing the relative virus content per plant. It can be concluded, therefore, that there is a rapid inactivation of alfalfa-mosaic virus in tobacco plants diseased for more than 12 days.

Tests for Presence of Neutralizing Antibodies

The rapid disappearance of virus activity after the 11th day is similar to that in some animal virus diseases and suggests that a defense mechanism in the tobacco plant may be causing an inactivation of virus in the plants. It seemed desirable, therefore, to examine diseased plants for the presence of neutralizing antibodies. Juice was extracted in the usual manner from

tobacco plants diseased for 10 and for 30 days and from healthy plants. Portions of each were ultracentrifuged for $1\frac{1}{2}$ hours at 30,000 r.p.m., and the upper third of the supernatant liquids was drawn off. Five-cc. portions of the supernatant liquids or of the original juices were mixed with an equal volume of the juice from plants diseased for 10 days. After standing for 1 hour at 4° C., the mixtures were used for inoculation. It may be seen from table 2 that in no case did the mixing of juice result in a striking reduction of activity.

TABLE 2

Results of Tests for Neutralizing Antibodies in Juice of Diseased Tobacco Plants

| Sample | Description | Lesions per half leaf ^a | | |
|-----------|-----------------------------------------------|------------------------------------|------|------|
| | | Un-diluted | 1:5 | 1:10 |
| 10 | Juice from plants diseased 10 days | 12.9 | 22.8 | 26.2 |
| 30 | Juice from plants diseased 30 days | 1.0 | 1.7 | 1.1 |
| H | Juice from healthy plants | 0.0 | — | — |
| HSL | Supernatant liquid from UC ^b of H | 0.0 | — | — |
| 10SL | Supernatant liquid from UC ^b of 10 | 1.0 | — | — |
| 30SL | Supernatant liquid from UC ^b of 30 | 0.1 | — | — |
| 10 + 30SL | Equal portions of 10 and 30SL | 5.1 | 9.9 | — |
| 10 + HSL | Equal portions of 10 and HSL | 8.8 | 15.9 | — |
| 10 + 30SL | Equal portions of 10 and 30SL | 5.2 | 8.3 | — |
| 10 + 10SL | Equal portions of 10 and 10SL | 7.9 | 11.8 | — |
| 10 + 30 | Equal portions of 10 and 30 | 4.0 | 12.1 | — |
| 10 + 10SL | Equal portions of 10 and 10SL | 4.5 | 16.1 | — |

^a The samples paired vertically were rubbed on 24 or more half leaves of *Phaseolus vulgaris* var. Early Golden Cluster. Dilutions were made with 0.1 M phosphate buffer at pH 7.1.

^b The term UC should read ultracentrifugation.

The ultracentrifuged juice from the older diseased plants had a slightly greater effect than that of healthy plants or of plants diseased for 10 days. On other occasions, the mixing of juices caused a reduction in activity of about the same order of magnitude. The experiments failed to demonstrate definitely the presence of antibodies in such juices. If such substances are present in the plants, they may be almost entirely neutralized by previously formed virus or the method used may destroy or fail to extract them. The fact that the reduction in activity is no greater with undiluted juice than with a 1:5 dilution indicates that the effect is not due to the presence of different amounts of an inhibitor in the juices.

Location of Virus in Old Plants

Since all leaves of plants that have been diseased for several weeks show no symptoms, it seemed desirable to determine the location of the small amount of virus in such plants. The leaves of 3 tobacco plants that had been diseased for 46 days were removed and divided into 4 groups, according to their location on the plants. The juice was obtained from each in the manner already described, diluted 1:10 with 0.1 M phosphate buffer, and used for activity measurements. The results are tabulated in table 3, together with notes on the appearance of the leaves included in each group. It is evident that about

TABLE 3
Location of Virus in Plants Diseased for 46 Days

| Location of leaves | Symptoms | Average weight of leaves per plant | pH of phosphate extract | Relative activity of juices ^a | Relative virus content ^b |
|-------------------------------------------------|-----------------------------------------------------------------------|------------------------------------|-------------------------|------------------------------------------|-------------------------------------|
| | | grams | | per cent | per cent |
| Upper 4 or 5 leaves plus tip | Faint mottling except in tips Very faint mottling. | 3.3 | 7.00 | 98 | 24 |
| Upper middle 3 or 4 leaves | Absent in some | 5.8 | 6.67 | 100 | 43 |
| Lower middle 6 or 7 leaves | No symptoms | 24.8 | 6.82 | 17 | 31 |
| Basal 4 or 5 leaves including inoculated leaves | No symptoms except for scattered primary lesions on inoculated leaves | 15.7 | 6.73 | 2 | 2 |

^a The figures in this column were obtained by dividing the number of lesions produced when a 1:10 dilution of the juice of the designated sample was rubbed on 24 or more half leaves of *Phaseolus vulgaris* var. Early Golden Cluster by the number produced when the control was rubbed on the opposite halves, and multiplying by 100. The control consisted of a 1:10 dilution of the sample to which the value of 100 is assigned.

^b The values in this column were calculated from the data in columns 3 and 5.

$\frac{3}{4}$ of the total amount of virus was located in the upper leaves, representing about 18 per cent of the total leaf weight. Since the older leaves were once high in virus content but at the time of cutting were very low, it can be concluded that virus had disappeared from these leaves.

Although most of the virus in old plants is located in the upper leaves, it does not reach so high a concentration there as it does in the leaves of plants inoculated for shorter periods of time. Activity measurements were made in the usual manner on juices collected from the upper leaves of old diseased plants and from corresponding ones of younger plants. It may be seen from table 4 that the juices from plants diseased for 7 and for 10 days were about 2 and 3 times as active, respectively, as that from the top of older plants. Hence, virus is not only lost from the older tissues but it does not reach a very

TABLE 4

Comparison of the Activity of the Juice from the Upper Leaves of Tobacco Plants Diseased for Long and for Short Periods of Time

| Days after inoculation | Location of leaves | Dilution | Lesions per half leaf ^a | Relative activity |
|------------------------|---------------------|----------|------------------------------------|-------------------|
| | | | | <i>per cent</i> |
| 7 | Upper 6 or 7 leaves | 1:10 | 49.5 | 100 |
| 51 | Upper 5 or 6 leaves | 1:10 | 24.0 | 58 |
| 7 | Upper 6 or 7 leaves | 1:50 | 10.0 | 100 |
| 51 | Upper 5 or 6 leaves | 1:50 | 6.7 | 68 |
| 10 | Upper 7 or 8 leaves | 1:10 | 60.9 | 100 |
| 51 | Upper 7 or 8 leaves | 1:10 | 20.3 | 33 |
| 10 | All 7 or 8 leaves | 1:50 | 14.2 | 100 |
| 51 | Upper 7 or 8 leaves | 1:50 | 1.8 | 13 |

^a The samples paired vertically were rubbed on 24 or more opposite half leaves of *Phaseolus vulgaris* var. Early Golden Cluster. Dilutions were made with 0.1 M phosphate buffer at pH 7.1.

TABLE 5

Concentration of Alfalfa-Mosaic Virus in Different Hosts at Intervals after Inoculation

| Host | Days after inoculation | No. of lesions per half leaf ^a | Relative activity |
|----------------------------|------------------------|-------------------------------------------|-------------------|
| | | | <i>per cent</i> |
| Necrotic-type tobacco | 11 | 30.7 | 100 |
| Turkish tobacco | 11 | 28.6 | 93 |
| Necrotic-type tobacco | 18 | 28.0 | 100 |
| Turkish tobacco | 18 | 23.7 | 85 |
| Necrotic-type tobacco | 33 | 15.4 | 100 |
| Turkish tobacco | 33 | 26.2 | 170 |
| Necrotic-type tobacco | 11 | 43.5 | 100 |
| <i>Nicotiana glutinosa</i> | 11 | 55.2 | 127 |
| Necrotic-type tobacco | 18 | 42.1 | 100 |
| <i>Nicotiana glutinosa</i> | 18 | 13.3 | 32 |
| Necrotic-type tobacco | 33 | 15.3 | 100 |
| <i>Nicotiana glutinosa</i> | 33 | 14.9 | 97 |

^a The diluted juices of the samples paired vertically were rubbed on 24 or more opposite half leaves of *Phaseolus vulgaris* var. Early Golden Cluster. They were diluted 1:10 with 0.1 M phosphate buffer at pH 7.1.

high level in those leaves formed after the 11th or 12th day following inoculation.

Virus Concentration in Other Hosts

Since only Holmes' necrotic-type tobacco was used in the foregoing tests, it became of interest to determine whether or not the virus behaved in a similar manner in other hosts. Young plants of *Nicotiana glutinosa* L., Turkish tobacco, and necrotic-type tobacco were inoculated with alfalfa-mosaic virus, and, at intervals, 3 plants of each were harvested and the juices extracted from each in the usual manner. It may be seen from the results presented in table 5 that there was a more rapid decrease in the virus content of *N. glutinosa* than in that of the necrotic-type tobacco; but, after 33 days, the concentration in the two hosts was about the same. Turkish tobacco plants, diseased for 33 days, contained more virus than those of the necrotic type. In general, however, the virus behaved in approximately the same manner in the 3 hosts and reached about the same concentration in each.

DISCUSSION

Tobacco plants inoculated with alfalfa-mosaic virus show the phenomenon referred to as recovery, for there is an acute stage of the disease followed by a chronic stage with much milder symptoms (3). This recovery differs in several respects from that in plants inoculated with tobacco-ring-spot virus (*Annulus tabaci* H. var. *virginiensis* H.) (6, 12). Plants that have recovered from tobacco ring spot show no symptoms except on those leaves where they had appeared before the recovery stage was reached. Plants recovered from alfalfa mosaic show symptoms only on the upper leaves, with the exception of the smallest leaves at the tip, and on the inoculated leaves. The symptoms on the upper leaves consist of a faint mottling, much milder than that apparent during the acute stage. The necrotic rings that sometimes appear on the inoculated leaf 4 to 7 days following inoculation do not entirely disappear. The systemic mottling characteristic of the acute stage gradually disappears and the leaves showing it become practically normal in appearance.

The changes in symptomatology of alfalfa mosaic are associated with the changes in virus concentration herein described. In some respects they resemble those observed by Price (6, 7) in plants diseased with tobacco ring spot; in other respects they differ. In both cases those leaves formed during the recovery period contain less virus and show milder symptoms than do leaves formed during the acute stage. In the case of tobacco ring spot, the virus content of those leaves showing symptoms during the acute stage remains fairly constant until the leaf turns yellow and drops from the plant. For a considerable period of time such leaves are higher in virus concentration than are the recovered upper leaves. In the case of alfalfa mosaic, the high concentration of virus reached during the acute stage is not maintained. As the symptoms disappear in the leaves, their virus content decreases. Consequently, the concentration of virus in the older leaves, once high, becomes

less than that in the upper leaves. Hence, the recovery of tobacco plants appears to be similar to that characteristic of plants affected with tobacco ring spot, with the added condition that alfalfa-mosaic virus is unstable *in vivo*. Prior to the 11th day, virus is produced at a more rapid rate than it is inactivated. The rapid decrease in the virus content after the 11th day may be due to a sharp decline in the rate of virus multiplication similar to that occurring with tobacco ring spot, while the rate of inactivation continues and exceeds the rate of multiplication, resulting in a net loss of virus. The cause of the inactivation is not known. The results of the few tests for neutralizing antibodies in the juice of recovered plants did not demonstrate their presence. It is possible that the decrease is due to a thermal inactivation *in vivo*, for the virus is unstable at greenhouse temperatures and the virus content of diseased plants appears to be less during hot summer months than in winter, when the temperatures were maintained at 20–24° C. (9).

SUMMARY

When young tobacco plants are inoculated with alfalfa-mosaic virus, the virus content of the plant increases from about the 4th to the 12th day after inoculation, then decreases rapidly. The juice from plants diseased for 48 days or longer may be less than 1 per cent as active as that from plants diseased for only 12 days. In the older plants, most of the virus is located in the upper leaves, which show only a faint mottling; but, even in such leaves, the concentration is much less than that reached about 12 days after inoculation. The older leaves, which once contained a high concentration of virus, contain very small amounts of virus and show no symptoms. Disappearance of symptoms is associated with loss of virus in such plants. It is suggested that tobacco plants, inoculated with alfalfa-mosaic virus, undergo a type of recovery similar to that exhibited by plants diseased with tobacco-ring-spot virus, and that the two phenomena differ in that the former virus is unstable *in vivo*.

The addition of dipotassium phosphate to ground diseased tobacco plants before pressing out the juice more than doubles the amount of virus extracted. With juice obtained in such a manner, dilution over a limited range results in an increase in the number of lesions produced. At medium dilutions, the number of lesions produced is inversely proportional to the dilution, but, at higher dilutions, the decreases in the number of lesions produced are much greater than would correspond to the changes in dilution.

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A STUDY OF PURIFIED VIRUSES WITH THE ELECTRON MICROSCOPE

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PLATES 1 TO 4

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Viruses were discovered in 1892 when Iwanowski (1) observed that the agent causing the mosaic disease of tobacco passed through a filter which retained all of the bacteria then known. During the ensuing years many viruses causing diseases in plants, animals, and bacteria have been discovered, and in general these agents have also been found to pass filters which retain ordinary bacteria. It has been necessary, therefore, to devise special means for determining the sizes of these very small infectious agents. For some years the method of ultrafiltration analysis with graded collodion membranes was widely and successfully used (2). Ultra-violet light photography (3), fluorescent microscopy (4), and special staining techniques (5) were also used for some of the larger viruses. By means of such methods it was established that the sizes of viruses ranged from about 250 $m\mu$ down to about 10 $m\mu$. Although objects as small as 5 $m\mu$ may be rendered visible by dark-field illumination, nothing may be gleaned as to their detailed structure; hence, it is obvious that ordinary microscopy cannot, in general, be used successfully for the viruses, since the limit of resolution for visual light is about 250 $m\mu$.

During the past 5 years, several viruses have been obtained in highly concentrated and presumably essentially pure form, and it has been possible to learn something of the size and shape of the particles in these preparations by means of sedimentation, diffusion, double refraction of flow, viscosity, and x-ray studies (6).

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One of the outstanding developments was the finding that, in confirmation of an earlier indication (7), some of the viruses depart markedly from a spherical form. For example, by means of data obtained in the studies mentioned above, tobacco mosaic virus was estimated by indirect methods to be about $12\text{ }\mu$ in diameter and about $400\text{ }\mu$ in length and to have a molecular weight of about 40 millions (8). Although the theory of the physico-chemical behavior of spherical particles has been worked out fairly thoroughly and appears to rest on a firm foundation, that of particles which depart markedly from a spherical form assuredly is on a much less firm basis. Frampton, for example, considers that in the case of tobacco mosaic virus the asymmetry and molecular weight values calculated from physicochemical data are wholly ambiguous and has shown that by his method of calculation a molecular weight value of infinity on the one hand and of zero on the other hand may be obtained (9). For reasons which have already been discussed (6), there appears to be little justification for the assumption of such an extreme view-point. Nevertheless, the indirect methods provide at best only approximations of the true sizes and shapes of asymmetrical particles and a means for the direct mensuration of such particles has been lacking.

In recent years electron microscopes having resolving powers extending down to about $5\text{ }\mu$ have been developed. Complete descriptions of the different instruments and of the mode of operation and preparation of specimens may be found elsewhere (10-13). Although excellent micrographs of bacteria have been obtained by means of this apparatus and have proved of value in supplementing information already available (11, 14), it would appear that the electron microscope will be of greatest value in the microscopy of objects having sizes between about 5 and $250\text{ }\mu$, a range not covered by the light microscope and one in which practically all viruses have been found to fall. The electron microscope offers the possibility of securing micrographs of individual virus particles and thus of establishing their sizes and shapes with some precision. It should also be possible to determine the extent of the variation in the size and shape of a given virus and even perhaps learn something of the mechanism by means of which a virus particle is duplicated within the host and of the

nature of the difference between strains of a given virus. In this paper are presented the results of preliminary electron microscopic studies of five plant viruses.

EXPERIMENTAL

Tobacco Mosaic Virus—Tobacco mosaic is the only one of the viruses used in the present study which has been investigated previously by means of an electron microscope (15-18). Most of the virus preparations used in the earlier studies were purified by chemical treatment and such treatment has been found to cause inactivation and aggregation of this virus. Tobacco mosaic virus purified by means of differential centrifugation has been found to be essentially the same as the virus in the untreated infectious juice with respect to biological activity and physicochemical properties (19); hence it appeared desirable to repeat and extend the electron microscope studies with virus purified by differential centrifugation. In preliminary work a small drop of a solution containing 0.2 mg. of four times ultracentrifuged tobacco mosaic virus per cc. in distilled water was applied by means of a capillary pipette to a collodion film about 15 m μ thick supported on a copper gauze. An attempt was made to secure as thin a film of liquid as possible on the mount. The film was allowed to dry, the mount was placed in the microscope, and the chamber was evacuated. When an area near the center of the mount was brought into focus, the field shown in Fig. 1 (Plate 1) was obtained. It is obvious that the concentration of virus was too great to give a good definition of the individual particles. Fig. 2 presents the appearance of a field nearer the edge of the same mount, in which the individual particles may be seen. Still greater separation of the particles was obtained in an area near the edge, which is shown in Fig. 3. A similar area of another virus preparation which was applied in the same way but at a concentration of 0.01 mg. per cc. is given in Fig. 4 (Plate 2).

The virus shown in Figs. 1 to 3 was used about 3 weeks after preparation. It seemed possible that the granular background and the suggestion of a granular structure for the rods might result from some aging process. Freshly prepared samples of tobacco mosaic virus were applied at a concentration of 0.01 mg. per cc. and examined by means of the electron microscope. Some

of the results which were obtained are shown in Figs. 5 to 7 (Plates 2 and 3), and it may be seen that the granular appearance is absent in these micrographs. However, when a drop of dilute ammonia was added to 1 cc. of an aqueous solution containing 0.01 mg. of tobacco mosaic virus and the preparation immediately observed, the results shown in Figs. 8 and 9 were obtained. The rod-like particles begin to disintegrate with the formation of material which has a granular appearance. It is known from previous chemical work that an excess of alkali causes the denaturation and disintegration of tobacco mosaic virus (20). In Fig. 8 some of the rods may still be seen, whereas in Fig. 9 the field is free of rods and only a granular material remains. However, it is known from previous studies (11) that the film obtained from a dilute solution of an inorganic salt also has a granular appearance. The induced or the spontaneous disintegration of a virus preparation or the presence of a small amount of inorganic material may, therefore, be responsible for the presence of granules. Later micrographs of virus aged for a period of some weeks were similar to those shown in Figs. 5 to 7; hence a granular appearance does not appear to be an invariable result of aging.

Figs. 2 to 7 demonstrate unequivocally the existence of discrete rod-like units in purified preparations of tobacco mosaic virus. The fact that the bulk of the material exists in this form, together with the fact that a great mass of evidence has been accumulated which indicates that the virus activity is associated with such a unit (6), makes it reasonable to assume that the predominating unit shown in Figs. 3 to 6 represents a single particle of tobacco mosaic virus. During the past few years, indirect evidence was obtained which indicated that under certain conditions there occurred an end-to-end as well as a side-to-side aggregation of tobacco mosaic virus (21). Figs. 3 to 6 provide convincing evidence for the existence of elongated aggregates presumably formed by the end-to-end combination of two or more units. Side-to-side aggregation, as well as a combination of this with end-to-end aggregation, is shown in Figs. 5 to 7. The type of aggregation shown in Fig. 7 appears to be that which obtains in the structures which have been referred to as crystals of tobacco mosaic virus (20). There is little indication of a regular structure and, in accordance with earlier results (16), the mass has more

nearly the appearance of a fiber. However, x-ray data have been obtained which indicate that in such aggregates the rods are arranged laterally in two-dimensional, hexagonal close packing (22).

The nature of the forces involved in the end-to-end type of aggregation is of some interest. There is evidence that the ultimate unit of tobacco mosaic virus possesses a dipole moment in the direction of the long axis or that such a moment is induced by an electrical field (23, 24). However, an unsymmetrical distribution of specific charges may be responsible for the marked tendency of the particles to aggregate. The aggregates do not appear to represent the natural form of the virus, for when carefully prepared samples of virus or virus in the freshly expressed untreated infectious juice are examined by means of the analytical ultracentrifuge no evidence for the existence of the aggregates is obtained, whereas following treatment with salt the same samples show either a second sedimenting boundary, presumably due to a component formed by the end-to-end aggregation of two particles, or a more rapidly sedimenting diffuse boundary indicative of even more extensive aggregation. Furthermore, recent micrographs show clearly the unaggregated rods in the freshly expressed untreated juice from mosaic-diseased plants (18). It seems likely that much of the aggregation shown in Figs. 5 to 7 takes place at the time of the drying of the films, when a marked concentration of the virus occurs. As the final stages of the drying occur, a violent whipping motion has been observed by means of an ordinary light microscope. This may be responsible for the formation near the edge of the collodion film of such great masses as those shown in Figs. 5 and 6. The possibility of avoiding aggregation due to such causes through the use of more dilute solutions of virus is being investigated. However, the micrographs already obtained provide good evidence for the existence of a predominating unit having a fairly uniform size and shape. Many measurements of the dimensions of the unit seen in these micrographs have been made and the particle appearing in greatest preponderance is about $15\text{ m}\mu$ in diameter and about $280\text{ m}\mu$ in length. The precision in the measurements of particle lengths in this work is of the order of $5\text{ m}\mu$, while the absolute error in magnification may be as great as 10 per cent. As may be seen from Fig. 10, measure-

ments of the lengths of all of the 58 particles in two selected fields indicate that a unit having a length of $280\text{ m}\mu$ predominates. It may be calculated that on a weight basis over 50 per cent of the material exists in the form of particles having a length of $280\text{ m}\mu$ and over 70 per cent in the form of particles having lengths within 7 per cent of this value. The values for the dimensions of this unit do not conflict with estimates based on x-ray data (22) of a particle diameter of $15\text{ m}\mu$ and a particle length of some value

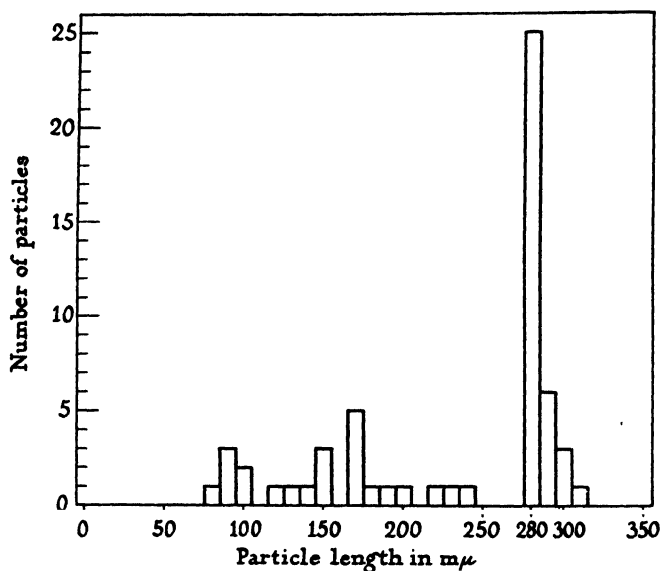


FIG. 10. Distribution of lengths of particles in an ultracentrifugally prepared sample of tobacco mosaic virus.

greater than $150\text{ m}\mu$. It seems likely that the value of $15\text{ m}\mu$ estimated from x-ray data and that of $280\text{ m}\mu$ estimated from the present micrographs represent the best values for the dimensions of the virus used in the present work. The density of tobacco mosaic virus has been found to be 1.33 (23, 25). The molecular weight of a particle having a circular cross-section $15\text{ m}\mu$ in diameter, a length of $280\text{ m}\mu$, and a density of 1.33 would be 39.8×10^6 . This value is in unusually good agreement with the value of 42.6×10^6 which was estimated by indirect methods and used tentatively in earlier calculations (21). It has been suggested

that the molecule collapses on drying so that the cross-section is elliptical rather than circular and that the molecular weight is actually slightly lower than the above. However, x-ray data on dried films indicate that there is no extensive collapse of the particles, for there is no distortion of the intramolecular structure and the interparticle distance in such films is 150 m μ (22). In addition, it may be possible to secure further evidence by refined techniques designed to determine the molecular weight from the total electron scattering produced by the molecule. However, it is apparent from the present micrographs that tobacco mosaic virus has a definite size, shape, and molecular weight and that the dimensions indicated by the electron microscope studies are of the same order of magnitude as those indicated previously by indirect methods (21). This finding is of importance in connection with the theories of the physicochemical behavior of asymmetrical particles, for it indicates that the indirect methods based on physicochemical data are reasonably valid when correctly used. Tobacco mosaic virus has therefore been of considerable value in demonstrating the usefulness of different methods of approach in the estimation of the size and shape of colloidal particles.

The particle length of about 280 m μ indicated by the micrographs of the ultracentrifugally purified tobacco mosaic virus used in the present work is significantly larger than the values of about 140 and 190 m μ which were estimated by Melchers and coworkers (18) from electron micrographs of two strains of tobacco mosaic virus, one of which is referred to as tomato mosaic virus since it was first noted in tomato plants. These results indicate that the strains of a virus have different particle lengths. Some years ago it was found in this laboratory that the sedimentation constants of different samples of the same strain of tobacco mosaic virus prepared from different lots of the same as well as different species of diseased plants were the same, whereas preparations of strains of tobacco mosaic virus even when obtained from the same type of host plant were found to have different sedimentation constants (26, 27). For example, when determined under the same conditions the sedimentation constant of the strain known as aucuba mosaic virus was found to be about 6 per cent larger than that of ordinary tobacco mosaic virus. Although at that time it was impossible to assign a definite reason for this difference, it was inferred that the difference was due either to a difference in

weight or to a difference in asymmetry. Because of the electron micrographs and the x-ray data which are now available, it seems likely that the particles of strains of tobacco mosaic virus differ both in weight and in asymmetry. The best estimate of particle thickness is probably provided by the x-ray data which indicate that the three strains, ordinary tobacco mosaic, aucuba mosaic, and enation mosaic viruses, all have the same diameter; namely, $15\text{ m}\mu$. The electron micrographs show the two strains of virus used by Melchers and coworkers (18) to have particle lengths of about 140 and 190 $\text{m}\mu$, respectively, and the strain used in the present work to have a particle length of about 280 $\text{m}\mu$. It is of interest to correlate these dimensions with the sedimentation constants of these preparations. Unfortunately, it is not known whether the sedimentation constant values reported by Melchers and coworkers represent true and reproducible values, since there was no indication of repeated determinations. However, assuming these values to be correct, it follows from Lauffer's work (28) that the values which were each reported to be 180×10^{-13} cannot in fact be identical, for one constant was determined at a virus concentration of 2 mg. per cc., whereas a concentration of 3 mg. per cc. was used for the other. If it be assumed that the variation in sedimentation constant with concentration is similar to that which Lauffer found to obtain with his preparations of tobacco mosaic virus, it may be calculated that the sedimentation constant of 180×10^{-13} at a concentration of 3 mg. per cc. corresponds to a constant of 183×10^{-13} at a concentration of 2 mg. per cc. It may or may not be significant that the corrected value of 183×10^{-13} belongs to the strain having the longer particle length of 190 $\text{m}\mu$. Although the values which Melchers and coworkers reported for the sedimentation constants may be fortuitous, the results now available for strains of virus at a concentration of 2 mg. per cc. would indicate a correlation between the length of particle and sedimentation constant, for preparations having particle lengths of 140, 190, and 280 $\text{m}\mu$ have sedimentation constants of 180, 183, and 187×10^{-13} , respectively. If these results are treated in the manner described by Lauffer and Stanley (21), it may be seen that there is a good correlation and that it is in accord with theory. Similar calculations show that the sedimentation constant reported for the dimer formed by the end-to-end aggregation of two particles of length 190 $\text{m}\mu$ is in good

agreement with the theoretical value. If the considerations just discussed are valid, it may be predicted that aucuba mosaic virus, which has been reported to have a sedimentation constant about 6 per cent larger than that of the virus used in the present work, should have a particle length of about 330 m μ . It is obvious, however, that, although the results already obtained indicate that strains of a virus have the same thickness but differ in both weight and particle length, many more experimental data must be obtained before the full significance of the differences between strains may be realized.

More extended observations must be made in connection with the electron microscope studies in order to establish the nature of any artifacts which may result from the drying of the film of a virus preparation or the exposure to the electron beam. The fact that a micrograph taken with the first flow of electrons through a given specimen does not appear to differ from subsequent micrographs taken after longer exposure to the electron beam makes it seem unlikely that gross changes are caused by the electrons. However, the violent motion which takes place as the film dries or the extreme desiccation which occurs on evacuation of the chamber containing the mount may cause some alteration of the specimen. Although it seems very unlikely that these could cause any gross changes in the size and shape of the particles, it is to be hoped that more exact information concerning the nature and extent of any change will become available as the work progresses. It has already been pointed out that on intensive drying of films of tobacco mosaic virus the interparticle distance decreases only from 152 to 150 Å. (22), thus indicating but little shrinkage.

In Figs. 3 to 6 of the present paper, a number of particles are in evidence which are definitely shorter than the predominating unit. It is not known whether these short particles occur regularly in preparations of tobacco mosaic virus or are produced at the time the specimen is mounted. It seems unlikely that they are due to an image produced by a particle of ordinary length which is not lying flat, since surface forces would tend to flatten all the molecules. Furthermore, the particles shown in the micrographs have about the same density, a condition which could obtain only if the particles were lying flat so that uniform thicknesses would be traversed by the electrons. There is at present

no evidence either from activity measurements on the supernatant fluids obtained on ultracentrifugation or from measurements by means of the analytical ultracentrifuge for the existence of these particles. They may, however, possess no virus activity or represent but a small fraction of a preparation and hence not be demonstrable by these methods. The true nature and significance of these short particles is not known at present. If it can be proved that they are not an artifact, that they regularly occur in mosaic-diseased cells and do not represent a degradation product, it is conceivable that they may represent partially synthesized virus particles or viable as well as non-viable virus variants. Nothing is known of the mechanism by means of which a virus particle is duplicated, but it is possible that these particles may provide a clue. The evidence at hand provides no definite indication as to whether duplication is preceded by longitudinal growth and lateral division, lateral growth and longitudinal division, growth from a point, or by some cataclysmic event, although the first possibility might appear most reasonable. It is to be hoped, however, that future work will provide some evidence regarding the course of events during the process of duplication of a virus particle.

Cucumber Mosaic Virus 3—Cucumber mosaic virus 3 may be regarded as being rather unusual, since it has not been found transmissible to any plants except members of the Cucurbitaceae (29). Most plant viruses do not have such a narrow host range; tobacco mosaic virus, for example, has been transmitted to forty-six different species of plants representing fourteen widely separated families (30, 31). Despite the fact that cucumber mosaic virus 3 will not multiply in plants susceptible to tobacco mosaic virus and the latter cannot be transmitted to cucumber plants, the two viruses have been found to have very similar physical, chemical, and immunological properties (32).¹ Although the x-ray data indicate a particle thickness of $14.6\text{ }\mu$, a value which is considered to be significantly smaller than the value of $15\text{ }\mu$ for tobacco mosaic virus (22), it seems possible that cucumber mosaic virus may have arisen from tobacco mosaic virus through some fortuitous event. It was therefore of interest to determine whether the micrographs of the particles of the cucumber mosaic virus obtained with the electron microscope would

¹ Knight, C. A., unpublished work.

be similar to those of tobacco mosaic virus. The samples of cucumber mosaic virus 3 used were prepared by means of differential centrifugation by Dr. C. A. Knight. It is a pleasure to thank Dr. Knight for making these preparations and those described in the following section available to us.

An aqueous solution containing 0.1 mg. of the virus per cc. was mounted as previously described and the micrograph reproduced in Fig. 11 (Plate 3) was obtained. It is obvious that the virus solution was too concentrated, so it was diluted with 99 volumes of water. The micrograph obtained with the dilute solution is shown in Fig. 12 (Plate 1). It may be seen that this virus has a rod-like form and that the diameter is about the same as that of tobacco mosaic virus but that the end-to-end aggregation appears to be somewhat more marked than in the case of tobacco mosaic virus. It is possible that the latter may be due to the use of a solution at a slightly more acid reaction and this point is now under investigation. It may be seen from Fig. 13 (Plate 3) that the rod-like particles of cucumber mosaic virus form fibrous aggregates similar in appearance to those formed by tobacco mosaic virus. Although more extensive studies will be required to establish definitely the length of the particle, measurements on the micrographs already available indicate that cucumber mosaic virus 3 has a particle length of about 300 m μ . The micrographs show, therefore, that, in accordance with previously obtained chemical, physical, and serological data, the ultimate unit of cucumber mosaic virus 3 is similar in size and shape to that of tobacco mosaic virus.

Cucumber Mosaic Virus 4—The results obtained with an ultracentrifugally isolated preparation of cucumber mosaic virus 4 at a concentration of 0.01 mg. per cc. are reproduced in Figs. 14 and 15 (Plate 4). A marked tendency to aggregate end-to-end is also noteworthy in the case of this virus. The electron micrograph reproduced as Fig. 14 is very similar to that of cucumber mosaic virus 3 shown as Fig. 12. The result was not unexpected, for the two viruses are strains and have been found to have very similar general properties (32).¹ Although several particles of cucumber mosaic virus 4 about 300 m μ in length are shown, it is not possible to determine from the micrographs now available whether or not the two strains have different particle lengths.

Tomato Bushy Stunt Virus—Tomato bushy stunt virus has been

purified by chemical means (33) and by differential centrifugation (34) and obtained in the form of large rhombic dodecahedral crystals. During the course of these studies, evidence was obtained that the virus particles were essentially spherical in shape and had a diameter of about $26\text{ m}\mu$ (35). An aqueous solution containing 0.01 mg. of ultracentrifugally isolated bushy stunt virus per cc. was mounted and studied by means of the electron microscope. It may be seen from Fig. 16 (Plate 4) that the particles which are shown have essentially the size and shape indicated by other methods. The tendency of the particles to collect along a fold in the collodion membrane may be noted. Since the size of bushy stunt virus has been well established by different independent methods (34-36), the good agreement of the size of the virus estimated from the electron micrograph with that estimated previously by other methods is significant, for it may be regarded as an indication that no gross change in size occurs during the preparation of the mount.

Tobacco Necrosis Virus—Pirie and coworkers (37) purified tobacco necrosis virus by chemical methods and reported that crystalline and amorphous preparations having the same specific virus activity had sedimentation constants of 130×10^{-13} and 58×10^{-13} , respectively. However, Price and Wyckoff (38) found a sedimentation constant of 112×10^{-13} for tobacco necrosis virus purified by differential centrifugation. The size of this virus was estimated to be between 13 and $20\text{ m}\mu$ by ultrafiltration measurements (39) and by means of radiation studies (40). Since the two extreme values for the sedimentation constant might be considered to indicate a size between about 10 and $30\text{ m}\mu$, it is obvious that only the order of the magnitude of the size of tobacco necrosis virus is known. In the present study an aqueous solution containing 1 mg. of ultracentrifugally isolated tobacco necrosis virus per cc. was used and the electron micrograph reproduced as Fig. 17 (Plate 4) was obtained. It may be seen that the particles appear to be spherical in shape and have diameters of about $20\text{ m}\mu$.

The writers desire to thank Dr. V. K. Zworykin for his interest and encouragement during the course of the work. It is also a pleasure to thank Dr. L. Marton and Mr. J. Hillier for assistance and advice during the preparation of the micrographs shown in the present paper.

SUMMARY

Purified preparations of five viruses have been studied by means of the electron microscope. The electron micrographs of the ultracentrifugally isolated tobacco mosaic virus used in the present work showed a predominating unit about 15 m μ in width and 280 m μ in length and presumably representing single particles of this virus, together with aggregates formed by the end-to-end as well as side-to-side aggregation of this unit and a small amount of rods having shorter although variable lengths. The fact that the dimensions of this unit were of the same order of magnitude as those estimated previously by indirect methods based on physico-chemical data indicates that the latter procedures are useful and essentially valid even for asymmetrical particles when correctly used. Since the particle length of the virus used in the present work was significantly greater than those of two strains studied by other workers, it seems likely that strains of a virus may have different particle lengths. The electron micrographs of cucumber mosaic virus 3 and of its related strain cucumber mosaic virus 4 were very similar, showed a marked amount of end-to-end aggregation, and indicated that the ultimate units were similar in size and shape to that of tobacco mosaic virus. In the case of tomato bushy stunt virus, the micrographs showed spherical particles about 26 m μ in diameter, whereas with tobacco necrosis virus the results indicated that the particles were essentially spherical and about 20 m μ in diameter.

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EXPLANATION OF PLATES

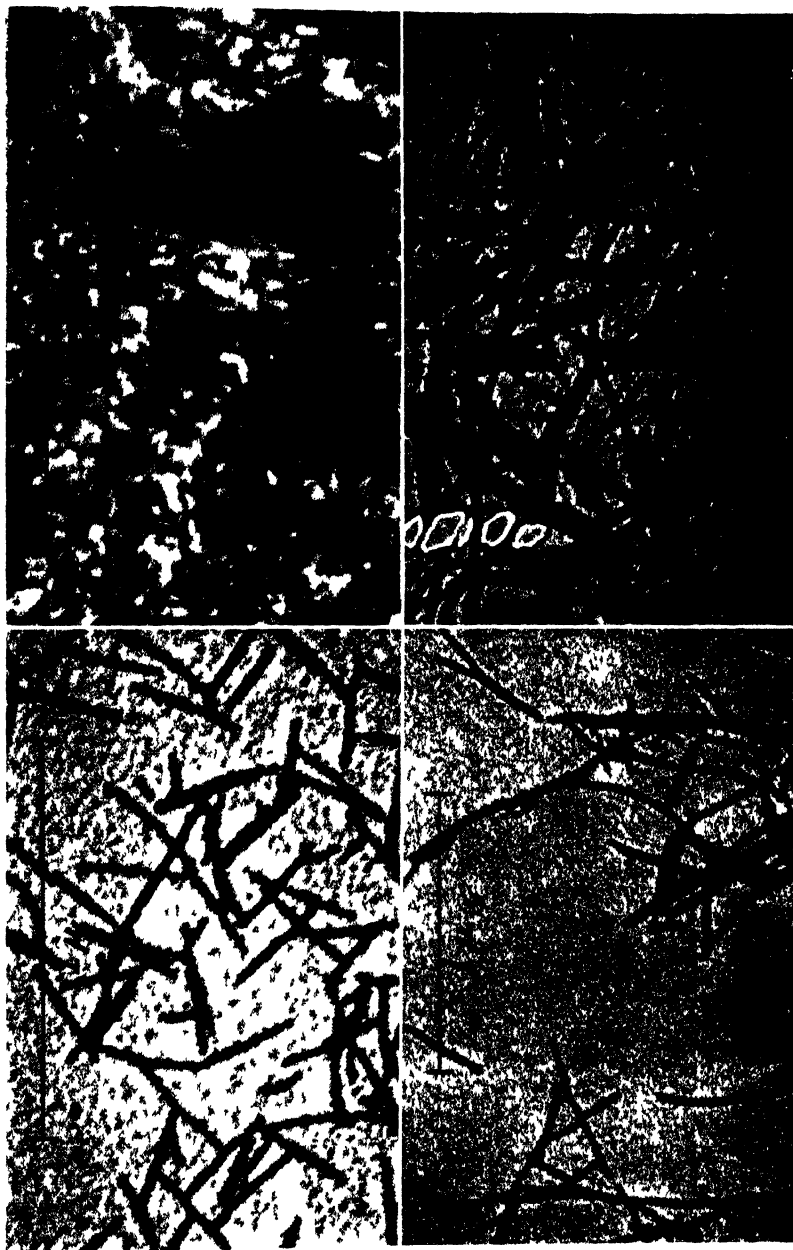
PLATE 1

FIG. 1. Area near center of mount prepared with an aqueous solution containing 0.2 mg. of ultracentrifugally isolated tobacco mosaic virus per cc. $\times 55,000$.

FIG. 2. Area nearer edge of mount used for Fig. 1. $\times 54,000$.

FIG. 3. Area near edge of mount used for Fig. 1. $\times 55,000$.

FIG. 12. Ultracentrifugally isolated cucumber mosaic virus 3 applied at a concentration of 0.001 mg. per cc., showing single particles and characteristic aggregation. $\times 39,000$.



(Stanley and Anderson Electron microscopy of viruses)

PLATE 2

FIG. 4. Tobacco mosaic virus applied to a collodion film at a concentration of 0.01 mg. per cc. $\times 24,600$.

FIG. 5. Tobacco mosaic virus applied at a concentration of 0.01 mg. per cc. Aggregation of particles near the fold in the collodion film may be noted. $\times 19,500$.



(Stanley and Anderson Electron microscopy of viruses)

PLATE 3

FIG. 6. Aggregation of tobacco mosaic virus near a fold in the collodion film as in Fig. 5. $\times 17,500$.

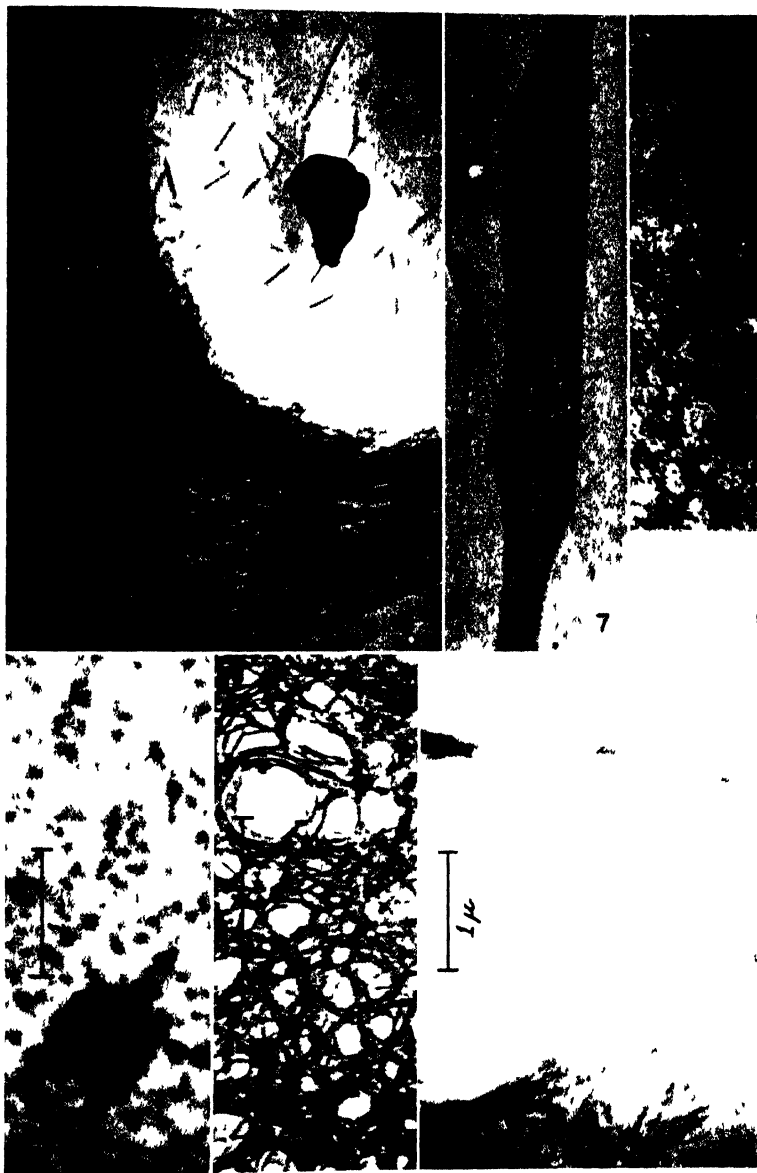
FIG. 7. Fiber-like aggregation of tobacco mosaic virus. $\times 22,500$. (Micrograph by Dr. L. Marton.)

FIG. 8. Partial disintegration of tobacco mosaic virus by dilute ammonia. $\times 11,250$.

FIG. 9. Complete disintegration of tobacco mosaic virus by dilute ammonia. $\times 17,500$.

FIG. 11. Ultracentrifugally isolated cucumber mosaic virus 3 applied at a concentration of 0.1 mg. per cc. A thick mat of virus and holes in the collodion film may be noted. $\times 25,800$.

FIG. 13. Fiber-like aggregate of cucumber mosaic virus 3. $\times 16,700$.



(Stanley and Anderson Electron microscopy of viruses)

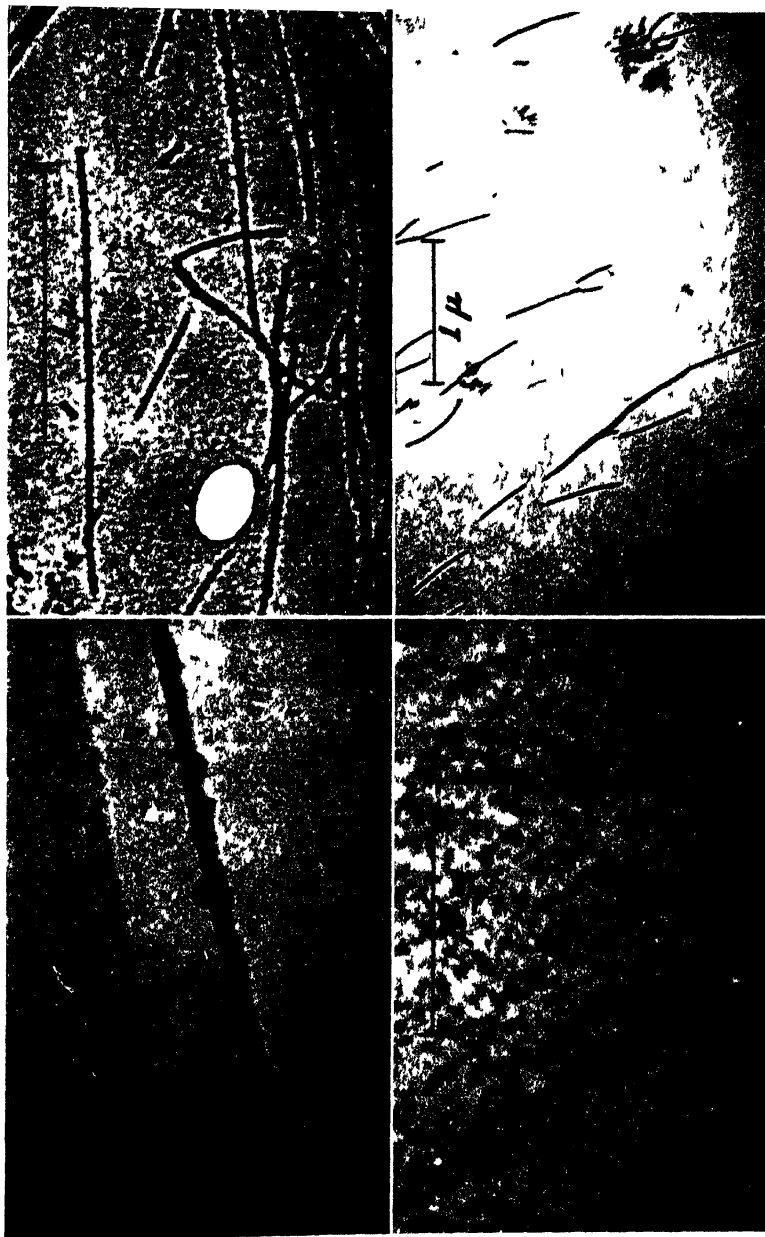
PLATE 4

FIG. 14. Cucumber mosaic virus 4 applied at a concentration of 0.01 mg. per cc. End-to-end aggregation of particles is especially noteworthy. $\times 39,000$.

FIG. 15. Cucumber mosaic virus 4. Several particles about 300 m μ in length are shown. $\times 20,000$.

FIG. 16. Ultracentrifugally isolated bushy stunt virus applied at a concentration of 0.01 mg. per cc. It may be noted that there is a tendency for the particles to collect at folds in the collodion membrane. $\times 31,600$.

FIG. 17. Ultracentrifugally isolated tobacco necrosis virus applied at a concentration of 1 mg. per cc. $\times 30,500$. (Micrograph by Dr. L. Marton.)



(Stanley and Anderson Electron microscopy of viruses)

A STUDY BY MEANS OF THE ELECTRON MICROSCOPE OF THE REACTION BETWEEN TOBACCO MOSAIC VIRUS AND ITS ANTISERUM

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PLATE 5

(Received for publication, February 3, 1941)

From the results of numerous experiments, immunologists have inferred that when a foreign material, usually protein in nature and called an antigen, is injected into an animal, it induces the formation of substances called antibodies, which appear in the animal's blood serum and which will react with the antigen injected (1-4). When this reaction results in the formation of a precipitate, it is called a "precipitin reaction." It seems likely that 1 antibody molecule is able to attach itself to more than one antigen particle and, *vice versa*, in such a manner that a framework or "lattice" of antigen particles is formed which gives rise to an antigen-antibody precipitate (1, 3, 5). On the basis of these ideas, explanations of the various phenomena of immunity have been advanced.

With the development of electron microscopes (6, 7) capable of recording the sizes, shapes, and reactions of protein molecules (8), it should be possible to observe certain of these immunological reactions directly. There are reported in the present paper the results of a preliminary study by means of the electron microscope of the reaction between tobacco mosaic virus and its antiserum. Tobacco mosaic virus was selected as the antigen because of its large size and distinctive shape. Many kinds of evidence indicate that the virus particles used in the present work are essentially cylindrical in shape and have lengths of about 280 m μ and

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diameters of about $15\text{ m}\mu$ (8, 9). Rabbit antiserum was selected as a source of antibodies to tobacco mosaic virus because the reactions involved have been extensively investigated (10) and because much information is available concerning the antibodies in rabbit sera (11, 12).

EXPERIMENTAL

Anti-tobacco mosaic virus rabbit serum was prepared and 1 cc. was added to 1 cc. of a solution containing 1 mg. of tobacco mosaic virus. It seems likely that in such a mixture the reaction would occur either in the equivalence zone or in the region of excess antibody (3, 10). The mixture was shaken and separate portions immediately diluted 1:10 and 1:100 with distilled water. After the preparations had stood for 1 hour at 25° , mounts of these solutions for the electron microscope were prepared in the usual manner (8) and electron micrographs were made of them. As a control, parallel preparations of tobacco mosaic virus alone and with added normal rabbit serum were made and micrographs prepared. After several hours a marked precipitate in the undiluted mixture of virus and antibody, a faint precipitate in the 1:10 dilution, and a faint Tyndall effect in the 1:100 dilution were observed. Mounts of these preparations were then made and observed by means of the electron microscope.

In Fig. 1 is presented the micrograph from the suspension containing 0.01 mg. of tobacco mosaic virus per cc. It may be noted that the molecules stand out sharply¹ and have widths of about $15\text{ m}\mu$ and lengths of about $280\text{ m}\mu$.

Fig. 2 is a micrograph of a mixture of tobacco mosaic virus and normal rabbit serum diluted 1:100 with distilled water. Here again the tobacco mosaic virus molecules stand out sharply with the normal lengths and widths, and with but few particles from the serum adsorbed on them. The contaminating bacterium

¹ It is convenient in obtaining micrographs of viruses to focus the electron microscope on relatively large and heavy particles. The pictures of these particles then reveal the order of resolution. This procedure is possible because of the great depth of focus of the electron microscope. Thus, the sharpness of the black particles in Fig. 1, of the bacterium in Fig. 2, of the edge of the collodion membrane in Fig. 3, and of the particle in Fig. 4 attests to the fact that the virus particles in all cases are in sharp focus and that the variations in widths which are observed are real.

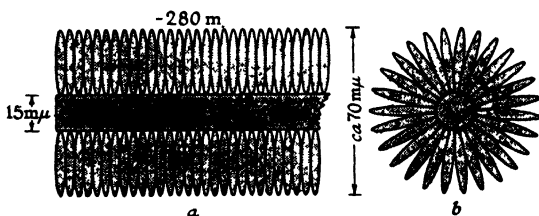
which may be observed in this figure serves to give a good idea of the relative size of the particles of tobacco mosaic virus. It may be seen that some of the tobacco mosaic virus molecules are adsorbed at right angles to the surface at the end of the bacterium. When mixtures of tobacco mosaic virus with precipitating rabbit antisera of approximately equal potency to tomato bushy stunt, potato latent mosaic, and tobacco ring spot viruses were used, similar results were obtained and there was no indication of an increase in the dimensions of the molecules of the tobacco mosaic virus.

Fig. 3 is a micrograph of the mixture of tobacco mosaic virus and antiserum diluted 1:100, which had stood for 1 hour. It is seen at once that the particles of tobacco mosaic virus appear at much greater contrast and are 3 or 4 times as wide as in the former preparations. This indicates that particles from the antiserum have become attached to the tobacco mosaic virus molecules, making them appear wider and at the same time presenting a thicker specimen to the electron beam. It is to be concluded that serum antibody molecules attach themselves at a great many points distributed over the surface of the tobacco mosaic virus molecule.

Fig. 4 shows a typical portion of the preparation of tobacco mosaic virus plus antiserum after standing for several hours at a dilution of 1:100. It may be seen that the particles are still extremely thick and, although the field is in sharp focus,¹ the particles appear fuzzy. Thus, there is a density gradient at the edge of the particles which may be due to the radial distribution of asymmetrical particles on the antigen. It is known that tobacco mosaic virus antibodies produced in rabbits are much smaller than the virus molecules,² and it seems likely that their dimensions are approximately the same as those of the antibody globulins of antipneumococcus rabbit sera which are considered to be about 3.7 $m\mu$ in diameter and 27.4 $m\mu$ in length (11-13). If a single layer of molecules having approximately these dimensions were to become attached laterally to a molecule of tobacco mosaic virus, the increase in diameter would be only about 8 $m\mu$ or an amount insufficient to explain the increase in width noted in Fig. 4. If, however, the molecules become attached to the virus molecule

² Stanley, W. M., unpublished work.

by their ends in a radial manner similar to that shown in Text-fig. 1, then the increase in width would be of the order of $55\text{ m}\mu$, or an amount sufficient to explain the increase in width noted in Fig. 4. It is also obvious from Text-fig. 1 that an electron beam passing through such an aggregate at right angles to the direction of the length would encounter regions of decreasing density as the edge was approached. An arrangement of asymmetrical molecules, such as that shown in Text-fig. 1, could therefore account for the indefinite and fuzzy appearance of the edges of the particles shown in Figs. 3 and 4. Furthermore, such a radial arrangement would be in accord with certain aspects of a recent theory of the structure and reactions of antibodies (5). It may also be seen from Fig. 4 that the antibodies seem to have joined the antigen molecules together to give at least the outward appearance of the



TEXT-FIG. 1. Possible mode of attachment of small asymmetrically shaped molecules to the side surface of a molecule of tobacco mosaic virus. (a) Longitudinal cross-section; (b) lateral cross-section.

framework or "lattice" of immunological theory. The irregularity of the observed framework, if due to such a chemical interlinking of antigen and antibody, would justify the avoidance of the term "lattice" by Heidelberger (3) and by Pauling (5).

Although the virus molecules appear to be well covered, it is not possible to determine from the present micrographs whether or not the coverage is complete. The fact that the dimensions of the virus molecules were unchanged in micrographs of mixtures with antisera to other viruses indicates that the primary reaction under investigation is specific in nature. Whether or not any non-specific serum protein was subsequently adsorbed onto the antigen-antibody complex cannot be determined until purified antibody preparations are used. Confirmatory evidence of the specificity of the primary antigen-antibody reaction was obtained in a study by means of the ultracentrifuge in which it was found

that the sedimentation constant of tobacco mosaic virus was essentially unchanged in mixtures containing an excess of normal rabbit serum or antisera to bushy stunt, ring spot, or latent mosaic viruses.² It was, of course, impossible to determine the sedimentation constant in the case of a mixture containing an excess of anti-tobacco mosaic virus serum, for the virus was completely precipitated and only the boundary due to the globulin fraction was apparent. In marked contrast, the sedimentation constant of bushy stunt virus was essentially unchanged in a mixture with anti-tobacco mosaic virus serum. The lack of reaction between bushy stunt virus and anti-tobacco mosaic virus serum was also demonstrated by means of electron micrographs.

The electron microscope thus makes it possible to observe directly the result of the reaction of particles in antiserum with antigen molecules and to record the nature of the precipitate which results from this interaction. The present results demonstrate the usefulness of the asymmetrically shaped tobacco mosaic virus and the advisability of making extensive studies of the antigen-antibody reaction by means of the electron microscope.

The authors are indebted to Dr. Michael Heidelberger, Dr. Stuart Mudd, and Dr. V. K. Zworykin for many helpful suggestions.

SUMMARY

1. Electron micrographs of tobacco mosaic virus deposited on a collodion film show that the molecules are about 280 $m\mu$ long and about 15 $m\mu$ wide.

2. Micrographs of a mixture of virus and normal rabbit serum show virus particles of normal size and indicate little or no adsorption of particles from normal serum onto the virus molecules. Similar results were obtained with mixtures of tobacco mosaic virus with antisera to tomato bushy stunt, potato latent mosaic, and tobacco ring spot viruses.

3. A mixture of tobacco mosaic virus and tobacco mosaic virus antiserum from rabbits, when dried on a collodion film an hour after mixing and examined by means of the electron microscope, shows particles about 60 $m\mu$ wide, about 300 $m\mu$ long, and having fuzzy profiles. The increase in particle width and the fuzzy appearance are regarded as indicating that the ends of asymmetri-

cally shaped molecules from the serum react specifically with the antigen molecules. No reaction between anti-tobacco mosaic virus serum and bushy stunt virus was demonstrable.

4. When the mixture of antigen and antiserum is applied to a collodion film several hours after mixing, an irregular framework of thickened antigen molecules may be seen. It is this framework which makes up the antigen-antiserum precipitate. The results demonstrate the usefulness of the electron microscope and of a large and distinctively shaped antigen such as tobacco mosaic virus in the study of the antigen-antibody reaction.

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EXPLANATION OF PLATE 5

FIG. 1. Ultracentrifugally isolated tobacco mosaic virus applied to a collodion membrane at a concentration of 0.01 mg. per cc., showing particles having a width of about 15 μ . $\times 13,700$.

FIG. 2. Mixture of same tobacco mosaic virus preparation used for Fig. 1 at a concentration of 1 mg. per cc. with an equal volume of normal rabbit serum and applied at a 1:100 dilution. The width of the virus particles is unchanged. $\times 13,800$.

FIG. 3. Mixture of same tobacco mosaic virus preparation used for Figs. 1 and 2 with an equal volume of an anti-tobacco mosaic virus rabbit serum and applied at a dilution of 1:100 to the collodion film 1 hour after mixing. Particles appear more dense and are about 60 μ wide. $\times 13,200$.

FIG. 4. Virus-antiserum mixture of Fig. 3 applied at a dilution of 1:100 several hours after mixing. Note that thickened virus particles have fuzzy profiles and have formed an irregular framework. $\times 13,700$.



(Anderson and Stanley: Antigen-antibody reaction)

SOME EFFECTS OF IODINE AND OTHER REAGENTS ON THE STRUCTURE AND ACTIVITY OF TOBACCO MOSAIC VIRUS

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The SH groups of denatured egg albumin give a pink^a color with nitroprusside (Heffter, 1907; Arnold, 1911) and reduce porphyrindin (Kuhn and Desnuelle, 1938). Native egg albumin does not give these characteristic SH reactions. Despite the fact that native egg albumin does not reduce porphyrindin, the SH groups of egg albumin or their precursors can be abolished by reaction of the native form of egg albumin with iodine (Anson, 1940; 1941).

Tobacco mosaic virus is an SH protein of the egg albumin type, since denatured but not native tobacco mosaic virus gives a pink color with nitroprusside and reduces porphyrindin (Stanley and Lauffer, 1939) and since the SH groups of tobacco mosaic virus, as shown by the present experiments, can be abolished by reaction of the native form of the virus with iodine. Iodine is the only reagent known to abolish the SH groups of egg albumin and tobacco mosaic virus by reaction with the native form of these proteins. The observations that denatured tobacco mosaic virus has SH groups and that these groups or their precursors can be abolished by reaction of the native form of the virus with iodine suggested the study of the effect of iodine on the activity of tobacco mosaic virus. It was found in the present work that the SH groups of tobacco mosaic virus can be abolished by iodine without any change in the activity of the virus, as shown by the number of lesions produced by a given amount of modified virus on *Nicotiana glutinosa* plants or by the symptoms produced in Turkish tobacco plants.

Tobacco mosaic virus has been inactivated by many different reagents, some of which are known to modify specific protein groups (Stanley, 1940). In the case of inactivation by formaldehyde, it was shown that the inactivation was accompanied by abolition of amino groups and that removal of formaldehyde was accompanied by an increase in free amino groups and by

partial reversal of the inactivation (Ross and Stanley, 1938). Although other viruses have been inactivated by formaldehyde, the structural changes brought about by formaldehyde were not established (Stanley, 1940). The formaldehyde experiments are the only ones in which the inactivation of tobacco mosaic virus has been associated with definite changes in protein groups by chemical tests on the modified protein. In no case has it hitherto been shown that tobacco mosaic virus or any other virus can be modified structurally by chemical procedures *in vitro* and still produce disease.

In the study of enzymes several cases have been found in which the enzyme structure can be altered without inactivation of the enzyme. The amino groups of pepsin can be acetylated without loss of the proteolytic activity (Herriott, 1934). Carboxypeptidase is active even in the presence of formaldehyde (Anson, 1937). Some of the SH groups of urease can be oxidized without any change in the urease activity (Hellerman, 1939).

The inoculation of Turkish tobacco plants with virus whose SH groups have been abolished with iodine results in the production of virus with the normal SH content. If the virus in the inoculum is not reduced within the living plant cells to virus with a normal SH content it must be concluded that iodine-modified virus causes the production, not of exact replicas, but of normal or unmodified virus. Such a result might be expected if virus with a normal SH content represents the nearest structure to the modified virus which may be synthesized within the plant cells due perhaps to some preexisting pattern. If, however, the inoculation of modified virus is followed by its reduction within the living plant cells to virus with a normal SH content it would be expected that the latter would stimulate the production of more normal virus. At the present time it is not known which of these alternatives represents the true course of events. It was found, however, that iodine-treated virus is not reduced to normal SH virus by a mash of normal tobacco plants. Unfortunately this is not absolute proof that such reduction does not take place in the living plant cells. Nevertheless it seems likely that reduction to SH of groups oxidized beyond S-S does not occur within the cells and hence that the present experiments provide an example in which a virus has been altered structurally without perpetuation of the structural changes in subsequent generations. Although at the present time it is impossible to assign definite reasons for the failure to perpetuate the structural change the results are important in connection with any consideration of the mechanism of virus reproduction.

If enough iodine is added to egg albumin (Anson, 1941) or to tobacco mosaic virus, not only are the SH groups abolished but the tyrosine groups are converted into di-iodotyrosine groups. When enough iodine is added

to tobacco mosaic virus to iodinate the tyrosine groups, the virus is inactivated. This result does not of itself prove that the inactivation is due to the change in the tyrosine groups and not to some other iodine reaction which occurs under similar conditions. However, it is known that when enough iodine is added to insulin (Harrington and Neuburger, 1936) or pepsin (Herriott, 1937) to iodinate the tyrosine groups, these proteins are likewise inactivated.

Whether or not it is possible to convert some of the tyrosine groups of tobacco mosaic virus into monoiodo or di-iodotyrosine groups without inactivating the virus is not decided by the present experiments.

Iodoacetamide at pH 8.0 under the conditions used in the present experiments abolishes few if any SH groups of tobacco mosaic virus, but nevertheless almost completely inactivates the virus. This result is of some interest for iodoacetamide has been regarded as a specific reactant for protein SH groups. It is hoped that the nature of the reaction which results in the inactivation by iodoacetamide will be elucidated in future work.

The present experiments raise such questions as whether all viruses, like tobacco mosaic virus, have SH groups which react with iodine but not with porphyrindin; whether in all cases oxidation of the SH groups fails to cause irreversible inactivation or indeed any change in the general character of the disease or in the type of virus produced in the infected plant; and whether all viruses can be inactivated by concentrated iodine and iodoacetamide. The fact that a virus variant was not produced by changes such as those described in the present paper does not mean that the production of variants by chemical treatment is impossible. It may be that the production of chemical variants must await the development of techniques for changing the amino acid content or arrangement of a virus without causing loss of virus activity, rather than merely changing amino acid groups such as SH. Experiments of the kind which have been done with tobacco mosaic virus in which the changes in both protein structure and activity are followed can, in case of necessity, be carried out with only a few milligrams of purified virus. It is to be hoped that similar experiments will be carried out with different viruses and also that different reactions will be used in the attempt to produce virus variants *in vitro* by definite chemical changes of protein structure.

EXPERIMENTAL

The Nitroprusside Test.—The nitroprusside test used in the present experiments is carried out as previously described in a solution of guanidine hydrochloride prepared from purified guanidine carbonate (Anson, 1941).

With recrystallized egg albumin about the same pink color is obtained with nitroprusside, whether 1 drop of 0.1 M cyanide is added or not. This small amount of cyanide suffices to combine with heavy metal impurities and does not cause any significant reduction of S-S to SH. With some samples of tobacco mosaic virus, however, an extremely weak nitroprusside test is obtained unless a drop of dilute cyanide is added. This indicates that some samples of tobacco mosaic virus may contain impurities which interfere with the nitroprusside test for SH groups.

The cyanide-nitroprusside test for S-S groups which are reduced to SH by cyanide is conveniently carried out as previously described (Anson, 1941) by adding 1 drop of 2 N cyanide to the protein in strongly alkaline guanidine hydrochloride solution and adding the nitroprusside 5 minutes later. The nitroprusside test carried out with no cyanide or with dilute cyanide which does not reduce S-S will be referred to as the nitroprusside test. When strong cyanide which can reduce S-S is added, the test will be called the cyanide-nitroprusside test.

SH Titrations.—The SH groups of denatured egg albumin can be estimated by allowing the protein to stand 45 minutes in neutral guanidine hydrochloride solution and then determining how much porphyrindin must be added to abolish the nitroprusside test (Greenstein, 1938). This method was applied to tobacco mosaic virus, and it was found that 1 cc. of 0.0006 N porphyrindin was required for 10 mg. of virus in order to abolish the nitroprusside test (Stanley and Lauffer, 1939).

The SH titration in guanidine hydrochloride solution has recently been modified in two ways. First, ferricyanide, tetrathionate, and *p*-chloromercuribenzoate are used as titrating agents instead of porphyrindin. Second, the titrating agent is added before the guanidine hydrochloride instead of 45 minutes thereafter (Anson, 1941). When purified guanidine hydrochloride is used, the same SH titration value for egg albumin is obtained whether ferricyanide is added before or after the guanidine hydrochloride. When the guanidine hydrochloride happens to contain impurities—which almost all commercial samples tested were found to contain—then low results are obtained by the original procedure because some SH groups are oxidized while the protein is standing in guanidine hydrochloride solution before the addition of ferricyanide. When ferricyanide is added to tobacco mosaic virus 45 minutes after the guanidine hydrochloride, different results are obtained by titrating the SH groups of different samples of virus even when purified guanidine hydrochloride is used, because some samples of virus themselves contain impurities which bring about the abolition of SH groups in guanidine hydrochloride solution. When guanidine hydrochloride of suitable purity is used and the titrating agent is added before

the guanidine hydrochloride, then 1 cc. of 0.00056 N ferricyanide, tetrathionate, or mercuribenzoate is needed to abolish the nitroprusside test of 10 mg. of tobacco mosaic virus, and all samples of virus give the same titration value. In the following experiments, SH groups are estimated by the ferricyanide titration method with ferricyanide added before the guanidine hydrochloride according to the directions previously described (Anson, 1941).

The SH groups of egg albumin (Anson, unpublished results) and of tobacco mosaic virus can also be estimated by measuring the blue color obtained when the proteins reduce Folin's uric acid reagent in neutral urea solution. The values obtained agree with those obtained by the ferricyanide titration.

Ross (1940) found that tobacco mosaic virus contains no methionine and a total amount of SH plus S-S sulfur which, within the experimental error, accounts for the total sulfur content of the virus of 0.2 per cent. The total SH plus S-S was estimated by titrating the SH groups in an HI hydrolysate, in which any S-S has been reduced to SH. The present SH titrations in guanidine hydrochloride solution confirm earlier results (Stanley and Lauffer, 1939) and show that all the sulfur of tobacco mosaic virus can be accounted for by SH alone.

Reactions with Iodine.—Tobacco mosaic virus prepared by differential ultracentrifugation (Stanley and Wyckoff, 1937; Stanley, 1937) has been treated with iodine under various conditions and the products tested for SH groups, for groups which can be reduced to SH by cyanide, for tyrosine groups by the Millon test, and for virus activity. The results are given in Table I. In the first experiments small amounts of iodine are added to neutral tobacco mosaic virus at 0°C., all the iodine is consumed, and the minimum amount of iodine is found which abolishes the nitroprusside test in guanidine hydrochloride solution.

0.5 cc. of iodine solution (prepared by diluting a stock solution of 0.1 N I_2 in 0.18 N KI) is added to 0.5 cc. of 2 per cent tobacco mosaic virus plus 0.1 cc. of a buffer consisting of equal parts 1 M Na_2HPO_4 and 1 M NaH_2PO_4 . All the solutions are precooled in ice water. After this solution has stood 30 minutes at 0°C. and the solution gives no color with starch, the nitroprusside test in guanidine hydrochloride solution is carried out. The test is positive if the amount of iodine added is 1.5 times the amount theoretically needed to oxidize the SH groups found in denatured tobacco mosaic virus to S-S, and negative if 2.5 times the theoretical amount is added. Even when no test for SH groups is obtained, a strong test is obtained if the iodine-treated protein is first exposed to alkaline cyanide which can reduce S-S to SH.

If iodine is added to native egg albumin at 0°C. and in the presence of 1 N KI, the SH groups of egg albumin can be abolished by the addition of

1 cc. of 0.001 N iodine to 10 mg. of native egg albumin. This is exactly the amount theoretically needed for the oxidation of the SH groups in denatured egg albumin to S-S. If 2 cc. of 0.001 N iodine is added, then 1 cc. of 0.001 N iodine is consumed, as shown by back titration with thiosulfate (Anson, unpublished results). When 1 cc. of 0.00056 N I_2 (the theoretical amount) is added to 10 mg. of neutral tobacco mosaic virus at 0°C. and in the presence of 1 M KI, no iodine is used up in 30 minutes, as shown by back titration with thiosulfate.

TABLE I
Reactions of Iodine with Tobacco Mosaic Virus

| Composition of reaction mixture 0.5 cc. virus solution 0.5 cc. I_2 solution 0.1 cc. PO_4 solution | | Temperature | Time | Nitro- prusside test | Cyanide- nitro- prusside test | Millon test | Lesions per half leaf | | Esti- mated amount of inac- tivation* |
|----------------------------------------------------------------------------------------------------------------|------------------------------------|-------------|------|----------------------------|----------------------------------------|----------------|-----------------------------|------------------|---------------------------------------------------|
| Concentration of virus solution | Concentration of I_2 solution | | | | | | Iodine- treated virus | Control virus | |
| mg. per cc. | N | °C. | hrs. | | | | | | per cent |
| 20 | 0.00168 | 0 | 0.5 | — | + | + | 56.5 | 65.5 | 14 |
| 20 | 0.00280 | 0 | 0.5 | — | + | + | 70.0 | 79.0 | 11 |
| 10 | 0.01 | 37 | 2 | — | + | + | 35.7 | 35.4 | 0 |
| 10 | 0.04 | 37 | 2 | — | — | + | 29.1 | 29.2 | 0 |
| 10 | 0.04 | 37 | 8 | — | — | + | 79.2 | 104.5 | 24 |
| 10 | 0.04 | 37 | 24 | — | — | + | 35.8 | 46.0 | 22 |
| 10 | 0.05 | 37 | 2 | — | — | + | 28.8 | 34.8 | 17 |
| 10 | 0.06 | 37 | 2 | — | — | + | 25.4 | 32.2 | 21 |
| 10 | 0.06 | 37 | 2 | — | — | + | 31.2 | 47.7 | 35 |
| 10 | 0.03 | 55 | 0.5 | — | — | + | 15.3 | 30.1 | 49 |
| 10 | 0.04 | 55 | 0.5 | — | — | + | 11.6 | 22.0 | 47 |
| 20 | 0.1 | 37 | 2 | — | — | — | 2.5 | 30.3 | 92 |
| 10 | 0.1 | 37 | 2 | — | — | — | 4.5 | 34.5 | 87 |
| 10 | 0.05 | 60 | 1 | — | — | — | 0.0 | 20.2 | 100 |

* Differences of less than about 20 per cent are not regarded as indicating a significant difference in virus activity.

One cannot decide on the basis of our experiments alone why the SH groups of tobacco mosaic virus, unlike the SH groups of free cysteine and of egg albumin, do not react with dilute iodine in 1 N KI. It may be that the SH groups of tobacco mosaic virus or their precursors are less reactive than the corresponding groups of egg albumin. It is also possible that there are spatial obstacles to the ready formation of S-S groups in native tobacco mosaic virus (*cf.* Neurath, 1940).

One might suppose that the tyrosine groups of native egg albumin would react with dilute iodine in 1 N KI, even if the SH groups fail to react. Even free tyrosine, however, does not react with dilute iodine if the solution contains 1 N KI (Anson, unpublished experiments).

In the second series of experiments, enough iodine is added to abolish

the cyanide-nitroprusside test but not the Millon test, which is positive for tyrosine groups and negative for di-iodotyrosine groups. All the iodine is not absorbed, so the excess iodine is removed before the tests are carried out. When 0.05 N I_2 is added under the conditions chosen, the cyanide-nitroprusside test is negative, the Millon test is strongly positive, indicating that few tyrosine groups have been converted into di-iodotyrosine groups, and the virus activity is essentially unaffected as shown by the fact that the iodine-treated virus produces about as many lesions as an equal amount of untreated virus. As the amount of iodine added or the temperature is increased, the Millon test and the activity become weaker and insoluble protein is formed. Similar results can be obtained by adding 0.05 N I_2 , that is, without increasing the iodine concentration, if the reaction is carried out for a day instead of 2 hours or if the solution is made more alkaline.

The experiments are carried out as follows. To 0.5 cc. of 1 per cent virus there are added 0.1 cc. of 1 M phosphate buffer at pH 6.8 and 0.5 cc. of iodine solution. The resulting solution is kept at the designated temperature and period of time in glass stoppered weighing bottles, then 0.5 cc. of thiosulfate of the same concentration as the iodine is added, and finally the mixture is made up to 5 cc. with water. For the color tests the protein is precipitated with 0.2 N trichloroacetic acid, centrifuged, stirred up with 0.2 N trichloroacetic acid, and centrifuged again. For the activity measurements, the solution is diluted 10 times with 0.1 M phosphate buffer at pH 7.0 and the virus activity compared with that of an equal amount of control virus by the half-leaf local lesion method on 20 or more leaves of *Nicotiana glutinosa* (Loring, 1937). The control virus is kept under the same conditions in the absence of iodine and at the end of the reaction tetrathionate instead of thiosulfate is added. At the virus concentrations used there is a direct proportionality between the virus activity and the number of lesions produced on *Nicotiana glutinosa* leaves although differences less than about 20 per cent in the lesion count are usually not regarded as indicating a significant difference in virus activity (Loring, 1937).

Although HI is a strong reducing agent, cysteic acid (RSO_3H) is not reduced to SH by HI under the conditions of the Baernstein HI hydrolysis of proteins (Kassell, 1940). Even when 0.04 N iodine is used to oxidize the SH groups of tobacco mosaic virus beyond the S-S stage, as shown by a negative cyanide-nitroprusside test, the protein on being dialyzed and then hydrolyzed with HI still yields as much cysteine as protein not treated with iodine. The oxidized groups are still reduced to SH by HI. This shows that the SH groups oxidized by iodine beyond the S-S stage are not oxidized as far as RSO_3H . We are indebted to Dr. A. F. Ross for carrying out the HI hydrolysis and estimating the cysteine content of the HI hydrolysate.

In the final experiment, a 1 per cent solution of virus is treated with an equal volume of 0.05 N iodine at 60°C. for 1 hour. The cyanide-nitroprusside and the Millon tests are negative, and the virus is completely inactivated. By carrying out the reaction at 60°C. instead of at 37°C.,

one avoids the formation of insoluble protein. If the protein is insoluble, one cannot be sure that the inactivation is due to the chemical change and not to the insoluble state of the protein.

SH Groups of Virus Produced in Plants Infected with Iodine-Modified Virus.—The following experiments show that the inoculation of Turkish tobacco plants with virus whose SH groups have been abolished by iodine is followed by the production of virus with the normal number of SH groups.

For the first experiment Turkish tobacco plants were infected with the virus partially inactivated by treatment with 0.06 N iodine at 37°C. as already described. Virus was isolated from the plants after they had been infected for 6 weeks and the SH of the virus was titrated with ferricyanide. The first time this experiment was carried out, virus was obtained whose SH groups were abolished by half the amount of ferricyanide normally required. When this experiment was repeated several times, however, the virus isolated from plants infected with iodine-treated virus always gave the normal ferricyanide titration value. Furthermore, tobacco plants infected with the virus which gave the low titration value also yielded virus with the normal titration value. It is not known why in one case and in only one case virus with a low titration value was obtained. It is possible that this one sample of virus became accidentally contaminated with impurities which interfered with the estimation of the SH groups.

The possibility existed that the iodine-treated virus preparation used to infect Turkish tobacco plants for the production of more virus contained a very small amount of virus which had escaped reaction with iodine, that this normal virus multiplied in the Turkish tobacco plants much more rapidly than the modified virus, and that the normal virus finally obtained had its origin in the small amount of normal virus which had escaped reaction with iodine. In one series of experiments, therefore, the iodine-treated virus was used to infect not Turkish tobacco, in which tobacco mosaic virus causes a systemic infection, but *Nicotiana glutinosa*, in which tobacco mosaic virus causes local lesions. When sufficiently dilute virus is used to infect such plants, each lesion is believed to contain only a single strain of virus, a fact which makes possible the separation of different strains of virus (Jensen, 1933; Kunkel, 1934). Turkish tobacco plants were infected by means of inocula prepared from single lesions previously obtained by rubbing iodine-treated virus over the leaves of *N. glutinosa* plants. The virus isolated from these Turkish tobacco plants was found to have a normal SH content.

In the first experiment, tobacco mosaic virus was treated with an equal volume of 0.01 N iodine for 2 hours at 37°C. as in the previous experiment with 0.04 N iodine. The

resulting virus gave a negative nitroprusside test but a positive cyanide-nitroprusside test. When tested against an untreated sample at a dilution of 10^{-4} gm. per cc. by the half-leaf method, the iodine-treated preparation gave an average of 35.7 lesions per half leaf and the control an average of 35.4 lesions per half leaf. If the lesions produced by the inoculation of iodine-treated virus had been caused only by virus which had escaped reaction with iodine, the number of lesions would have been greatly reduced by the iodine treatment. The virus treated with 0.01 N iodine was then used at a dilution of 10^{-6} gm. per cc. to inoculate the entire area of four leaves of a *Nicotiana glutinosa* plant. Five discrete and well separated lesions were selected and each was removed, macerated, and used as an inoculum for a group of four Turkish tobacco plants.

In a second experiment seven groups of four Turkish tobacco plants were infected by means of inocula prepared from seven single lesions obtained from virus almost completely inactivated by 0.1 I₂ instead of from virus not inactivated at all by 0.01 N I₂. It is extremely unlikely that this preparation contained any virus which had not reacted with iodine.

To 20 cc. of 2 per cent tobacco mosaic virus there were added 4 cc. of 1 M phosphate buffer at pH 7.4 and 20 cc. of 0.1 N iodine in 0.18 N potassium iodide. The mixture was kept at 37°C. for 2 hours, 20 cc. of 0.1 N thiosulfate was added, and the final solution was dialyzed overnight against cold distilled water in a shaking dialyzer. A precipitate of insoluble material was removed by centrifugation and found to contain 170 mg. of protein. The supernatant solution contained 0.2 mg. of virus per cc. which when applied to half leaves at a concentration of 10^{-4} gm. per cc. gave an average of only 2.5 lesions per half leaf. Since the untreated starting material when applied at a concentration of 10^{-4} gm. per cc. gave an average of 30.3 lesions per half leaf on the other halves of the same leaves the soluble fraction was about 90 per cent inactivated. The largely inactivated soluble fraction of the virus treated with 0.1 N iodine was used at a concentration of 10^{-4} gm. of protein per cc. to inoculate the entire area of four leaves of a *Nicotiana glutinosa* plant, in order to obtain the single lesions used to infect Turkish tobacco plants.

The character of the lesions produced by virus treated with 0.01 N or 0.1 N iodine and the course of the infection were the same as those observed when *Nicotiana glutinosa* plants are infected with untreated virus.

After 5 weeks the groups of Turkish tobacco plants infected from single lesions produced by virus treated with 0.01 N and 0.1 N iodine were cut and frozen as well as a group of Turkish tobacco plants inoculated at the same time with untreated virus. Several samples from each of the three groups were macerated and the virus isolated by the procedure involving differential centrifugation customarily employed in this laboratory. In all cases the virus finally isolated had the normal SH content as measured by ferricyanide titration and the normal specific activity as measured by the number of local lesions produced on half leaves.

The experiments which have been described show that the disease caused by virus whose SH groups have been abolished by iodine is not due to residual virus which escaped reaction with iodine. They show further that the iodine treatment does not produce a new variant. So far as one can tell by the tests used, iodine-treated virus brings about normal infection and the production of normal virus. The results do not permit a decision as to whether or not iodine-treated virus is reduced in the living plant to

normal virus before multiplication of virus takes place. It was proved, however, that a mash of Turkish tobacco plants does not reduce virus which has been oxidized by iodine.

The virus added to macerated Turkish tobacco leaves was treated with an equal volume of 0.04 N I_2 for 2 hours at 37°C., as previously described. After the addition of thiosulfate to destroy the excess iodine and dialysis, it gave a negative cyanide-nitroprusside test and a positive Millon test. 30 mg. of the iodine-treated, dialyzed virus was added to 20 gm. of a mash prepared by macerating by means of a meat grinder the fresh leaves of a normal Turkish tobacco plant. The mixture was allowed to stand overnight at room temperature and the juice was expressed and subjected to the purification process involving differential centrifugation customarily employed in this laboratory. The 18 mg. of virus which was isolated was found to give a negative cyanide-nitroprusside test.

TABLE II
Effect of Iodoacetamide on the Activity of Tobacco Mosaic Virus

| Concentration of virus | Concentration of iodoacetamide | Temperature | Time | Lesions per half leaf | | Estimated amount of inactivation |
|------------------------|--------------------------------|-------------|------|----------------------------------|---------------|----------------------------------|
| | | | | Virus treated with iodoacetamide | Control virus | |
| mg. per cc. | M | °C. | hrs. | | | per cent |
| 0.5 | 0.05 | 37 | 2 | 13.3 | 24.2 | 45 |
| 0.5 | 0.05 | 37 | 18 | 0.6 | 23.2 | 97 |
| 5 | 0.1 | 37 | 6 | 2.7 | 12.9 | 79 |
| 5 | 0.1 | 55 | 4 | 1.1 | 10 | 89 |

Reactions with Iodoacetamide.—Iodoacetamide at pH 9.0 abolishes 40 per cent of the SH groups of native egg albumin (Anson, 1940). Iodoacetamide (prepared according to Anson, 1939) was added to tobacco mosaic virus in 0.1 M phosphate buffer adjusted to pH 8 with NaOH, and the concentrations of the reagents and the time and temperature of the reaction were varied as shown in Table II. More alkaline solutions were not used in order to avoid inactivation of the virus by alkali. The virus in aliquot portions of the various preparations treated with iodoacetamide was precipitated and washed with trichloroacetic acid, dissolved in neutral guanidine hydrochloride solution, and titrated with ferricyanide. In every case the titration value was the same, within 10 per cent, as that obtained from normal untreated virus. In all the cases the virus was partially inactivated by iodoacetamide. The exact degree of inactivation, as shown in Table II, depended on the exact conditions of the reaction and in one case was as high as 97 per cent.

In a report (Anson, 1940) of some preliminary experiments, it was stated

that tobacco mosaic virus could absorb iodine without being inactivated and that neither tobacco mosaic nor rabbit papilloma virus was inactivated by iodoacetamide. In the present experiments inactivation was brought about by the use of much more concentrated iodoacetamide.

Non-Inactivation by p-Chloromercuribenzoate.—*p*-Chloromercuribenzoate, an SH reagent introduced by Hellerman (1939), combines with the SH groups of denatured egg albumin and denatured tobacco mosaic virus. It combines with native egg albumin either not at all or very loosely (Anson, 1941). In the present investigation it was found that 0.1 per cent tobacco mosaic virus is not inactivated at room temperature in a neutral solution containing 0.001 N mercuribenzoate, an amount which would combine with all the SH groups of the virus if the virus were denatured.

SUMMARY

1. Denatured tobacco mosaic virus has a number of SH groups corresponding to its total sulfur content of 0.2 per cent. The SH groups were estimated by titration with ferricyanide, tetrathionate, and *p*-chloromercuribenzoate in guanidine hydrochloride solution and by reduction of the uric acid reagent in urea solution.

2. The SH groups of tobacco mosaic virus or their precursors can be abolished by reaction of the native form of the virus with iodine.

3. Tobacco mosaic virus whose SH groups have been oxidized beyond the S-S stage by iodine but whose tyrosine groups have not been converted into di-iodotyrosine groups still retains its normal biological activity as shown by the number of lesions it causes on *Nicotiana glutinosa* plants and by the characteristic disease produced in Turkish tobacco plants.

4. The inoculation of Turkish tobacco plants with active virus whose SH groups have been abolished by iodine results in the production of virus with the normal number of SH groups.

5. If enough iodine is added to tobacco mosaic virus or if the iodine reaction is carried out at a sufficiently high temperature, then the tyrosine groups are converted into di-iodotyrosine groups and the virus is inactivated.

6. Tobacco mosaic virus can be almost completely inactivated by iodoacetamide under conditions under which iodoacetamide reacts with few if any of the protein's SH groups.

7. Tobacco mosaic virus is not inactivated by dilute *p*-chloromercuribenzoate.

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